VOLUME 7 July 1958 NUMBER 4

# THE AMERICAN JOURNAL OF

# Tropical Medicine and Hygiene

*Combining The American Journal of Tropical Medicine and The Journal of the National Malaria Society*

OFFICIAL ORGAN OF
THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

PUBLISHED BIMONTHLY BY
THE WILLIAMS & WILKINS COMPANY
BALTIMORE 2, MARYLAND, U.S.A.

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THE AMERICAN JOURNAL OF

# Tropical Medicine and Hygiene

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*Correspondence concerning business matters*, subscriptions and orders for reprints, should be addressed to The Williams & Wilkins Company, Mount Royal and Guilford Avenues, Baltimore 2, Maryland, U. S. A.

*Manuscripts and Books for Review* should be sent to Dr. Martin Frobisher, 374 Ruckman Road, Closter, New Jersey.

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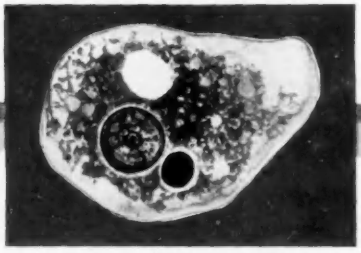

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1. Sodeman, W. A., and Beaver, P. C.: *Am. Jour. Med.*, 12:440, Apr., 1952.
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THE AMERICAN JOURNAL OF
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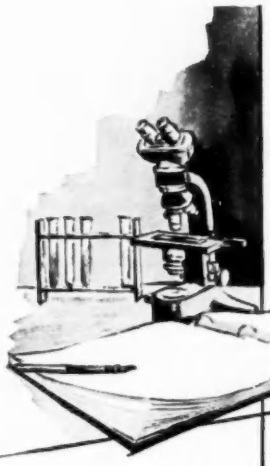

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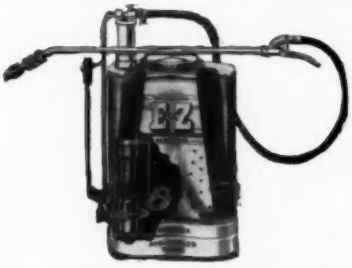

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# PRESENTATION OF THE JOSEPH AUGUSTIN LEPRINCE AWARD FOR 1957

JUSTIN M. ANDREWS

*National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service, United States Department of Health, Education and Welfare, Bethesda 14, Maryland*

To refresh the memory of those of you not familiar with the Joseph Augustin LePrince Award, I am reading a brief description of it which was contained in the first issue of "Tropical Medicine and Hygiene News" for this year, after which I shall have a few words to say concerning the recipient of this year's award.

"In his presidential address before the National Malaria Society in 1948 Dr. E. H. Hinman suggested 'the propriety of establishing an annual lectureship or the award of a medal commemorating the outstanding achievements of a pioneer in the field of malariology.' In 1949 the Committee on Lectureship and Award recommended the Joseph Augustin LePrince Award and the Award was established in that year by the National Malaria Society. Upon amalgamation of the National Malaria Society and the American Society of Tropical Medicine the Award was transferred to the new Society and the composition of the Award Committee was provided for in Article XII of our by-laws.

"The first Award was presented in 1951 to Mr. Joseph Augustin LePrince. In 1954 the Award was presented to Professor Brian Maegraith.

The conditions of the Award are as follows:

1. The Award consists of:
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   b. A certificate.
   c. A bronze medal.
2. 'Outstanding accomplishment in the field of malaria' is the only condition to eligibility for the Award.
3. The selection of the person to receive the Award is made by the Committee on the Joseph Augustin LePrince Award consisting of the President of the Society for the year of the Award (Chairman), the immediate Past-President, and the President-Elect for the year of the Award.
4. The presentation of an address upon receipt of the Award is optional for the recipient.

"The Council of the Society is pleased to announce that the bestowal of the Joseph A. LePrince Award in 1957 has been made possible by the Shell Chemical Corporation which has agreed to provide the honorarium and a sum to defray traveling expenses of the recipient of the Award."

The recipient for 1957 is Dr. Louis L. Williams, Jr., member of a family which has a probably unique history of unbroken career connection with the U. S. Public Health Service. The official records show that Dr. Louis Laval Williams, father of tonight's recipient, left private practice to join, in 1885, what was then known as the Marine Hospital Service. Two of his sons, Charles L. and Louis L., Jr., studied medicine and both of them entered the Public Health Service. Dr. Charlie, as he was generally known, retired in 1951, but he had prudently arranged to have the family Service association carried on by his son Charles, now on duty in the organization. Thus, for 71 consecutive years there has been a member of this family in the Regular Corps of the Public Health Service.

Dr. Louis L., Jr., joined it in 1913. He was a great admirer of Dr. Henry Rose Carter, of yellow fever and malaria fame, and early in his career Dr. Louis interested himself in malaria control. In 1926 he demonstrated the usefulness and economy of airplanes for wide-scale distribution of Paris green, then used as an anopheline larvicide over large areas of aquatic vegetation. The next year he was placed in charge of the malaria investigations of the Public Health Service with headquarters at Richmond, Va., later transferred to Washington, D. C. Under his guidance the malaria-control investigations of the Service played an important role in the industrial development of the South.

I first knew Dr. Williams in the National Malaria Society in the early '30's. At that time Dr. Francis Metcalf Root and I were offering at the Johns Hopkins School of Hygiene and Public Health a course in malariology. Both of us were somewhat more academic than practicing malariologists and so, to give the aspect of

verisimilitude to our students, we generally presented to our class each year at least one lecturer who was currently engaged full-time in the actual practice of malaria control; usually it was Dr. L. L. Williams, Jr.

In 1935 the Social Security Act was passed. This made funds available to State and local health departments in the malarious South to recruit and utilize malaria-control teams comprised of personnel trained to measure and prevent malaria. I have always suspected that Dr. Louis had something to do with my going to the Georgia Department of Public Health for this purpose in 1938.

In 1939 Dr. Williams gave leadership to a group of technical specialists loaned by the Service to the Chinese Government to advise regarding the suppression of malaria along the Burma highway.

During World War II the Public Health Service was requested to assist the Army in solving the problems of malaria control within the military establishments located in the South where many of the training camps and maneuver areas were established. Accordingly, in 1941, Dr. Williams was detailed as Liaison Officer at the Headquarters of the Fourth Service Command of the Army in Atlanta, Ga., because the majority of the malarious areas of the United States were included in the coverage of this Service Command. Dr. Williams drafted the plan of field organization for malaria control in military areas and assisted in the formulation of military policies for malaria control.

Shortly thereafter he was directed to expand the malaria-control activities carried on under military auspices by extending the zones of coverage into the civilian areas surrounding military establishments. In the implementation of this mission, Dr. Williams established an Office of Malaria Control in War Areas in Atlanta, bringing together professional and technical personnel with special competences in the various phases of malaria control. Control projects were operated by State health departments through State Offices of Malaria Control in War Areas and these were advised, coordinated, and supplied by the Federal organization established by Dr. Williams. Following World War II, this organization was converted, with expanded activities and responsibilities, into the Communicable Disease Center.

In 1943 Dr. L. L. was sent by General George Catlett Marshall, then Chief of Staff, as a special malaria envoy to General Dwight David Eisenhower, then Commanding General of the U. S. Army in the North African Theater of Operations. While he was overseas, a paper written by him was presented by the late Dr. Joseph Mountin before the National Malaria Society in which he advocated and set forth the principles of malaria eradication as a logical objective of antimalarial activities. This concept is one which Dr. Williams still propounds tirelessly and eloquently at every opportunity.

During the early postwar period it became apparent to the State Department that the health of people overseas had an important bearing on economic recovery in many of the countries receiving assistance from the United States. So, in 1945, Dr. Williams was assigned as medical advisor for duty with the Division of International Labor, Social, and Health Affairs of the State Department. Three years later he returned to the Public Health Service to operate the Division of International Health, organized previously by Dr. James A. Doull. This Division in effect consolidated the overseas health activities and interests of the Department of State and of the then Federal Security Agency.

Dr. Williams retired from the Public Health Service in 1953 and has since been a Consultant Medical Officer in the Pan American Sanitary Bureau where his lively interest in malaria control and eradication continues unabated.

This life-time dedication to the downfall of malaria simplified the selection of this year's recipient of the Joseph Augustin LePrince Award; the Committee unanimously named Dr. L. L. Williams, Jr.

Dr. Williams, it gives me great personal pleasure and official satisfaction to bestow upon you in the name of the American Society of Tropical Medicine and Hygiene the third Joseph Augustin LePrince Award for outstanding achievements as a pioneer in the field of malariology.

# INFLUENCE OF VERY LOW-FAT DIETS, WITH AND WITHOUT GLUTEN, ON THE ENDOGENOUS-FECAL-FAT EXCRETION OF PATIENTS WITH TROPICAL SPRUE

CONRADO F. ASENJO, R. RODRÍGUEZ-MOLINA, M. CANCIO AND R. A. BERNABE

*Department of Biochemistry and Nutrition School of Medicine, School of Tropical Medicine, University of Puerto Rico and the Medical Research Laboratory, San Patricio Veterans Administration Hospital, San Juan, Puerto Rico*[1]

So far as we know, controlled fat-balance studies in tropical sprue on very low-fat diets have been carried out in only two cases and for short periods. Black, *et al.*, (1946) kept two tropical sprue patients for two, consecutive, 3-day periods on a diet containing 6 gm of fat a day and observed that by the second 3-day period the fat excretion was within normal limits. They concluded that the disappearance of steatorrhea on a very low-fat diet was strong evidence against intestinal secretion of fat playing an important role in producing this condition.

Dicke (1950) and also Dicke, *et al.*, (1953), and van de Kamer, *et al.*, (1953) point out that the administration of gluten to celiac patients on very low-fat diets resulted in a significant increase in endogenous-fecal-fat excretion, thereby introducing a new factor in the etiology of steatorrheas. Their observations were confirmed by such investigators as Anderson, *et al.*, (1952); Sheldon and Lawson (1952); Soderhjelm (1952); Gerrar, *et al.*, (1955); and Ross, *et al.*, (1955). All observed that a significant percentage of the celiac patients returned to a normal fat absorption in periods ranging from 30 to 50 days after entering a gluten-free diet; on the other hand, renewed administration of gluten resulted in a reappearance of steatorrhea. They concluded that the steatorrhea of celiac disease is really due in part, if not totally, to an increased excretion of endogenous fat.

The effect of gluten has also been studied in cases of nontropical sprue. McIver (1952) observed improved fat absorption in one patient put on a gluten-free diet. Anderson, *et al.*, (1954) found that seven of 12 patients with adult idiopathic steatorrhea showed no improvement on a gluten-free diet, but the remaining five patients showed remissions with a return of fat absorption to normal. They also placed three cases of tropical sprue on a wheat-free diet. After a period of 27 days the fat absorption in one case reverted to normal; in the other two, the persistence of severe diarrhea necessitated the adoption of additional therapy. They concluded that, in the small number of cases studied by them, wheat gluten does not appear to be a primary etiologic factor. Ruffin, *et al.*, (1954) reported clinical improvement in three patients with nontropical sprue on a wheat-free diet. Recently, Schwartz, *et al.*, (1957) studied the effect of a gluten-free diet on fat excretion in nontropical sprue. In two patients who underwent detailed metabolic balance studies, the percentage of fat absorbed returned to normal when gluten was eliminated from the diet.

The present study was undertaken to determine the level of endogenous fecal-fat excretion in patients with tropical sprue and severe steatorrhea, kept on a very low-fat diet with and without gluten.

## MATERIALS AND METHODS

Tropical sprue, with severe steatorrhea, is not infrequently encountered in the Puerto Rican male veteran, particularly in those over 40 years of age (Rodríguez-Molina, Asenjo and Cancio, 1957). Only those cases that met the diagnostic criteria of Rodríquez-Molina (1954) for tropical sprue were initially selected for further study. These diagnostic criteria include the following signs and symptoms:

1. insidious onset, chronicity of symptoms, and paucity of spontaneous remissions;
2. presence of stomatoglossitis, with or without atrophy of the lingual papillae;
3. gastrointestinal disturbances ("dyspepsia," diarrhea and steatorrhea);

[1] This investigation was carried out as a cooperative project under a grant from the U. S. Public Health Service, National Institute of Health Research Project A-889.

4. history of recent weight loss, accompanied by increasing weakness and prostration;
5. presence of free HCl in the stomach in over 90 per cent of cases;
6. presence of macrocytic, hyperchromic anemia, associated with megaloblastic marrow;
7. a flat glucose tolerance curve (oral);
8. absence of neurologic manifestations in most cases;
9. hyperpigmentation of the skin, particularly the face, arms and legs;
10. "inflammation" and atrophy of the gastric and rectosigmoid mucosa.

Fat-balance studies at different levels of fat intake are the only exact method of determining dietary fat absorption as well as endogenous fat excretion. In order to choose the best conditions for a well-controlled fat-balance experiment, we carried out a series of short-term preliminary trials with a basal low-fat-low-gluten diet which consisted of fruit juices, bananas, cooking bananas, potatoes, tropical root crops, skimmed milk, coffee, sugar and lean-beef meat or dried codfish. This diet was relatively well accepted by the patients. It supplied, at the most, 8 gm of fat per day and its gluten content was negligible.

During the preliminary trials, the acceptability rating of the low-fat-low-gluten diet, supplemented with gluten or with fat, was also tested. The low-fat-low-gluten diet supplemented with gluten involved the same dietary regime described above, except that four slices of gluten bread and a bowl of Cream of Wheat were added daily. A full serving of this diet supplied not more than 12 gm of fat and not less than 30 gm of gluten per day. It was also relatively well accepted by the patients.

On the other hand, when 40 gm of dietary fat (butter, vegetable oil and lard) was integrated into a daily serving of the low-fat-low-gluten diet the results were unsatisfactory, as the majority of the patients rejected the diet totally. The largest amount of fat that could be introduced without having the diet lose its acceptability was 20 gm per day; this amount, however, was not high enough to make it a practical steatorrhea test diet. It was therefore decided to adopt the Mayo Clinic (1954) steatorrhea test diet with some modifications. The latter consisted of fruit juices, dry breakfast cereals, Cream of Wheat, white bread, potatoes, three different salads of lettuce with different fruits and dressings, cooked rice, jelly, baked apple, water-packed fruits, string beans, carrots, one or two eggs, lean beef or lamb, butter, and one liter of milk per day. Three menus were made up from these food items which, if fully consumed, supplied between 90 to 103 gm of fat and about 11 gm of gluten per day.

The preliminary trials definitely indicated that although the patients accepted the low-fat-low-gluten diet comparatively well, a certain variation would be necessary to keep them for longer periods on this monotonous diet.

Three menus were therefore also made up from the ingredients already described for the low-fat-low-gluten diet. This permitted keeping the patients on the low-fat-low-gluten regime in the final experiment for as long as 24 days without complaint.

On the basis of the experience gained in these preliminary fat-balance trials, the schedule indicated below was worked out for the final experiment:

1. preliminary period on the steatorrhea test diet (no stool collections made), 2 days;
2. experimental period on the steatorrhea test diet, 6 days (two 3-day trials);
3. experimental period on the low-fat-low-gluten diet supplemented with gluten, 12 days (four 3-day trials)
4. experimental period on the low-fat-low-gluten diet, 12 days (four 3-day trials);
5. experimental period on the steatorrhea test diet, 6 days (two 3-day trials).

A dietitian specially assigned to this study prepared and served the food. This was weighed out before serving; any food residues left were also weighed. A whole day's serving was homogenized, and aliquots of this homogenate were analyzed for total fat and solids. In order to make the necessary corrections, samples of the residues left by each individual patient were also analyzed. The patients were kept under strict surveillance.

Stools were collected and weighed daily, stored in the ice box, and within 48 hours homogenized by the addition of a known amount of water, if necessary, and analyzed for total fat and solids.

The determination of total solids in diet and stools was accomplished by weighing about 20 gm of homogenate and drying the same to constant weight at a temperature of 90°C.

Dietary and stool total fat were determined by the method used by van de Kamer, *et al.*, (1949). Briefly, this consists of weighing a sample of homogenized wet sample not containing over 0.3 gm of fat. The sample is saponified after adding 10 ml of 33 per cent KOH in $H_2O$ and 40 ml of ethanol containing 0.4 per cent amyl alcohol. The mixture is then boiled for 20 minutes under a reflux condenser, and cooled. Seventeen ml of 25 per cent HCL are added, after which the mixture is again cooled; extracted with 50 ml of petroleum ether and shaken vigorously in the special Erlenmeyer flask set-up described by the authors of this method. After complete separation, 25 ml of the petroleum-ether layer are transferred to a small flask by pressure pipette. The petroleum ether is evaporated and 10 ml of neutral ethanol are added. The fatty acids are titrated with 0.1-N NaOH from a microburette, with thymol blue as indicator, until the yellow color begins to change. The calculations are made following the formula developed by van de Kamer, *et al.*, (1949), which expresses total fat in terms of total fatty acids of an average molecular weight of 284.

Total fatty acids, in gm, per 100 gm sample = $$\frac{A \times 284 \times 1.04 \times 2}{10{,}000\ Q^2} = 5.907\ A^2$$

Knowing the percentage of total fatty acids in the diet and in the feces, as well as the weight of diet ingested and feces excreted per day, the intake and output per day of total fat, in terms of fatty acids, were calculated.

Fat-balance experiments were also conducted on a group of normal male subjects maintained on a basic Puerto Rican rural diet supplying approximately 50 gm of fat per day. This diet was composed principally of cooked, polished rice, red kidney beans, tubers, lard, oleo, dried codfish, and a variety of other minor constituents.

All normal subjects were males between 18 and 25 years of age. They were carefully selected after submitting them to a thorough medical examination; all were free of intestinal parasites and belonged to a group being used for a nitrogen balance experiment. Having their records of food intake and fecal output, as well as homogenized samples of diets and feces, the fat content was determined by the procedure already described and the fat balance computed.

[2] A = ml 0.1-N alkali used in the titration; Q = gm of feces taken for analysis.

TABLE 1

*Preliminary trials: daily fecal-fat excretion of tropical-sprue patients on a low-fat diet with and without gluten. (Average values and standard deviation)*

| Number of subjects | Solids excreted per day (gm) | Fat ingested per day (gm) | Fat excreted per day (gm) |
|---|---|---|---|
| | With gluten | | |
| 8 | 41.5 ± 17.7 | 1.71 ± 0.88 | 1.22 ± 0.86 |
| | Without gluten | | |
| 9 | 44.1 ± 23.7 | 2.40 ± 2.10 | 1.30 ± 0.60 |

## RESULTS AND CONCLUSIONS

The preliminary trials on the low-fat-low-gluten diet, and on this same diet supplemented with gluten, lasted for periods of 3 to 8 days. The average 24-hour endogenous fecal fat excretion of the patients kept on these diets is recorded in Table 1. These preliminary, short-term trials not only provided information on managing the experimental subjects, acceptability of diets, etc., but also yielded valuable experimental data and gave a good indication that the daily endogenous-fecal-fat excretion of tropical-sprue patients was of about the same magnitude as that of normal subjects.

The patients chosen for the final experiment were screened by submitting them to the steatorrhea test diet for a period totalling 8 days; that is, a 2-preliminary-day period, and two 3-day trial periods. The results of this screening for steatorrhea are recorded in Table 2. The criteria suggested by Asenjo (1952) for assessing fat absorption were used.

Only those patients with an average daily fat output of more than 6 gm and an apparent coefficient of fat absorption[3] of less than 90 per cent were considered to be suffering from severe steatorrhea. Of the 11 patients screened, two had mild steatorrhea (J. A. and A. C.), but the remaining nine fulfilled our strict criteria for severe steatorrhea. These were the ones chosen for the final experiment in which the daily fecal-fat-output per day was measured on the low-fat-low-gluten diet, supplemented with gluten, and on the low-fat-low-gluten diet alone.

[3] Apparent coefficient of fat absorption = $$\frac{\text{dietary fat intake} - \text{fecal fat output}}{\text{dietary fat intake}} \cdot 100.$$

TABLE 2

*Fat balance studies in tropical-sprue patients on the steatorrhea test diet. (Average of 6 collection periods per patient following a 2-day preliminary period on the diet)*

| Patient | Dietary solids ingested per day (gm.) | Fecal solids excreted per day (gm.) | Dietary fat ingested per day (gm.) | Fat in dried feces (%) | Fat excreted per day (gm.) | Apparent absorption | |
|---|---|---|---|---|---|---|---|
| | | | | | | Solid (%) | Fat (%) |
| J. R. R. | 430 | 54.1 | 67.0 | 20.7 | 11.2 | 87.4 | 83.3 |
| E. V. | 485 | 50.3 | 57.2 | 30.2 | 15.2 | 89.6 | 73.4 |
| J. M. | 429 | 23.9 | 70.3 | 43.5 | 10.4 | 94.4 | 85.2 |
| J. A. | 468 | 23.7 | 86.6 | 32.9 | 7.8 | 94.9 | 91.0 |
| E. M. | 519 | 93.1 | 95.2 | 44.4 | 41.3 | 82.1 | 56.6 |
| H. P. | 429 | 49.0 | 71.1 | 35.3 | 17.3 | 88.6 | 75.7 |
| N. C. | 528 | 47.7 | 77.7 | 25.6 | 12.2 | 90.9 | 84.3 |
| A. C. | 518 | 28.5 | 81.8 | 24.2 | 6.9 | 94.5 | 91.6 |
| J. L. | 528 | 35.9 | 77.7 | 37.9 | 13.6 | 93.2 | 82.5 |
| E. S. | 429 | 48.0 | 71.1 | 40.4 | 19.4 | 88.8 | 72.7 |
| I. Q. | 575 | 42.3 | 82.2 | 49.6 | 21.0 | 92.6 | 74.5 |
| Average | 485 | 45.1 | 76.2 | 35.0 | 16.0 | 90.6 | 79.2 |

The fecal fat outputs of the nine patients chosen for the final trials are summarized in Figure 1. In the upper part of the diagram, the bars represent the dietary fat intake and those in the lower part, the fecal fat output. The whole bar represents the magnitude of the intake or output, and the dotted areas represent the standard deviation. Each bar represents the average of 27 observations, that is, three 24-hour trials for each of the nine patients studied.

The results indicate that the excretion of endogenous fecal fat while on the steatorrhea test diet was well above 6 gm in all cases. On the other hand, when fed the low-fat-low-gluten diet supplemented with gluten, there was a rapid decrease in the amount of fecal fat excreted until it reached a steady output of about 2 gm per day. When the gluten was eliminated from the diet and the low-fat-low-gluten diet was fed alone, the fecal fat output per day remained at the same low level during the 12 days that the trial lasted.

In contrast with these results, there was a gradual, but steady increase in fecal fat output with values well above 6 gm when the patients were again fed the steatorrhea test diet. According to the above results, endogenous fecal fat excretion in tropical sprue seems to be of about the same magnitude as in normal subjects; dietary gluten does not seem to have any effect on its excretion.

The data in Table 2 indicate that the magnitude of the fat absorptive defect among the sprue patients varies appreciably from case to case. For example, the values obtained for daily fecal fat excretion and per cent apparent fat absorption in cases J. A. and A. C., showed these two particular patients to be suffering a mild steatorrhea only, while the remaining nine cases yielded values characteristic of severe to very severe steatorrhea.

Table 3 records the results of the trials with normal subjects kept on an average Puerto Rican rural diet. Each trial lasted 8 days and 4 subjects were used per trial.

It is interesting to note that the daily fecal solid excretion in the normal subjects averaged 23.5 gm (range 22.0 to 26.8), while in the sprue patients it was almost twice as much; average 45.1 (range 23.7 to 93.1). However, the normal subjects ingested more dietary solids (594 gm per day) than the sprue patients (485 gm per day). The average apparent coefficient of solid absorption[4] for the normal subjects was 96.1 per cent (range 95.6 to 96.3) and for the sprue patients, 90.6 per cent (range 82.1 to 94.9). The average fecal-fat excretion of the normal subjects was 1.1 gm per day (range 0.6 to 1.4) and the coefficient of apparent fat absorption was 97.8 per cent.

When these results are compared with those

[4] Apparent coefficient of solid absorption $= \frac{\text{dietary solid intake} - \text{fecal solid output}}{\text{dietary solid intake}} \cdot 100.$

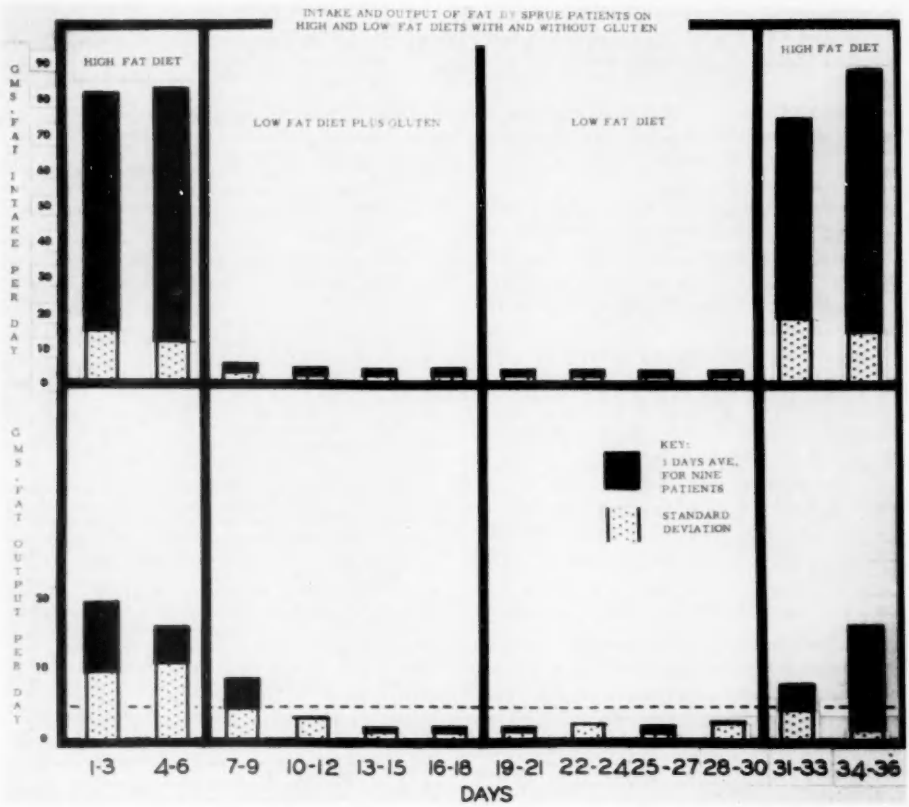
INTAKE AND OUTPUT OF FAT BY SPRUE PATIENTS ON HIGH AND LOW FAT DIETS WITH AND WITHOUT GLUTEN
HIGH FAT DIET
LOW FAT DIET PLUS GLUTEN
LOW FAT DIET
HIGH FAT DIET
GMS. FAT INTAKE PER DAY
GMS. FAT OUTPUT PER DAY
KEY:
3 DAYS AVE. FOR NINE PATIENTS
STANDARD DEVIATION
1-3 4-6 7-9 10-12 13-15 16-18 19-21 22-24 25-27 28-30 31-33 34-36
DAYS

FIG. 1. Dietary fat intake and fecal fat output of sprue patients on the dietary regimes indicated in the diagram.

obtained in normal subjects by other investigators, as compiled by Asenjo (1952), it can be noticed that our group of normals had an average excretion of only about one-fourth as much fecal fat per day and exhibited a somewhat higher apparent coefficient of fat absorption. These differences could possibly be explained on the basis of the intake of fat per day which, in our normal group was less than in other groups. Also, at least in the group reported by Asenjo (1952), the method used to determine total fecal fat was a gravimetric one which, in addition to the true fat, also included the unsaponifiable fractions that would tend slightly to increase the results.

TABLE 3

*Fat balance studies in normal subjects receiving a Puerto Rican rural diet (each trial is the average of 4 different subjects and 8 collection periods per subject. (Average values and standard deviation of the 7 trials)*

| Trials | Dietary solids ingested per day (gm) | Fecal solids excreted per day (gm) | Dietary fat ingested per day (gm) | Fat in dried feces (%) | Fecal fat excreted per day (gm) | Apparent absorption | |
|---|---|---|---|---|---|---|---|
| | | | | | | Solid (%) | Fat (%) |
| 7 | 594 ± 32 | 23.5 ± 1.9 | 51.9 ± 1.3 | 5.0 ± 1.2 | 1.1 ± 0.3 | 96.1 ± 0.2 | 97.8 ± 0.3 |

A third factor that could have influenced the results was the type of diet given to the normal group reported by us, which was a bland diet soup-like in consistency.

What ever could have been the reason, our normal subjects absorbed fat very efficiently.

SUMMARY

Fat-balance experiments on patients with tropical sprue and on normal subjects are reported.

All patients studied showed a steatorrhea of exogenous origin resulting from a fat absorption defect and not from an increased excretion of endogenous fecal fat. When these patients received a low-fat diet, their endogenous fecal fat excretion was of about the same magnitude as that of normal subjects.

It was also demonstrated that, under the conditions of this experiment, gluten does not seem to have any effect on the endogenous excretion of fecal fat by tropical-sprue patients.

ACKNOWLEDGMENT

The authors wish to acknowledge their indebtedness to Dr. José A. De Jesús, Chief of Section of Diseases of Gastrointestinal System at San Patricio Veterans Administration Hospital, who was in charge of the patients; to Professor J. A. Goyco for providing the data on food intake and fecal output, as well as the samples of diet and feces of the normal subjects; and last but not least, to our dietitian, Miss Matilde Dávila, for most efficiently managing the dietary aspects of this investigation.

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# ACQUISITION OF INTESTINAL PROTOZOA AND HELMINTHS BY YOUNG CHILDREN IN A TYPICAL VILLAGE OF LOWER EGYPT[1]

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In a rather extensive study of the diseases of the peoples of Egypt by U. S. Naval Medical Research Unit No. 3, a number of surveys were made to learn the incidence of intestinal protozoa and helminths. Although the majority of this work was based upon a study of villagers above the age of 5, several lots of material included younger children. The intestinal fauna and their incidence were quite different from those reported for adults (Lawless, *et al.*, 1956; Wells and Blagg, 1956 and Kuntz, *et al.*, unpublished data). For this reason it seemed desirable to learn which intestinal protozoa and helminths were present in young children whose parents were known to live under conditions favorable for parasite propagation. Furthermore, it was assumed that a weekly stool sample taken from very young children over a period of several months would indicate the approximate times at which the different intestinal fauna become established in a village population.

In conjunction with other studies now in progress by NAMRU-3 and the International Health Division of the Rockefeller Foundation (Weir, *et al.*, 1952) the present study was undertaken at Sindbis, a small village representative of the delta area of lower Egypt. Sindbis is located in Qalyubia province about 16 miles northwest of Cairo and is a typical village of farming people with a population of about 4200. The living and sanitary conditions in the community studied bear directly upon the parasitological findings. Description of these need not be repeated here since Weir, *et al.*, (1952) and Chandler (1954) have given these details concerning Lower Egypt as well as the village of Sindbis. Chandler studied the effects of improved sanitation upon parasite incidence in the population of Sindbis as compared to that of Aghour El Kubra, a neighboring village which received no assistance in improvement of living conditions and thus served as a control. Our work was carried out prior to and during the earlier stages of the improvement program set up for Sindbis.

[1] The opinions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department.

The authors are indebted to Rear Adm. J. J. Sapero, MC., USN., (ret.) Commanding Officer of NAMRU-3, Cairo, Egypt, who participated in the earlier phases of this study.

## MATERIALS AND METHODS

Over 100 children under the age of 4 years were selected somewhat at random from the village. In the beginning there were equal numbers of subjects in each of three arbitrary age groups: *Group I*, 1 to 6 months of age; *Group II*, children 13 to 24 months old; and *Group III*, those 26 to 47 months of age. As anticipated, due to movements of the parents, deaths, lagging interest on part of the parents, etc., a number of the children were dropped from the survey. However, sufficient stools were collected weekly for a period of 4 months to make the present report possible. In the final count there were 23, 30 and 28 children, respectively, in the three age groups.

The junior author, or a well trained and disciplined technician from the parasitology laboratory, made several trips a week to the village. Mothers cooperated surprisingly well in our weekly requests after becoming familiar with our project and having benefited from medical care given by physicians associated with this and other studies in the area. An attempt was made to reach the home of subjects from whom fecal specimens were desired at approximately the same hour on each visit. Fecal samples were removed from the cloths which served as diapers for the babies. Frequently mothers delayed the changing or cleaning of soiled "diapers" until the time of our arrival. Fecal specimens from older children were collected in sputum cups. The majority of samples were fixed within 1 to 2 hours after passage since all specimens were processed in the village as soon as obtained.

Several samples of feces the size of a garden pea were selected, placed in MIF (methiolate-iodine-formalin) (Sapero and Lawless, 1953) and were well macerated with an applicator stick in 15-ml capacity screw-cap vials. Vialed specimens

were studied later as opportunity permitted. A 10-to-15-minute search was made of a single direct smear consisting of several drops (22 x 22 mm cover slip) from a sample of the upper layer of sediment of each vial. This type of sample represents considerable concentration by the sedimentation principle, especially if the vial is allowed to stand at least an hour before the sample is withdrawn.

## SURVEY FINDINGS

The incidence of intestinal protozoa and helminths as detected in three different age groups of children is presented in Table 1. Data are supplemented by bars to demonstrate more clearly the increase in infection rates in the three different age groups of children studied for a period of approximately 4 months. The composite age span represented, although not great for the three groups, is sufficiently broad to emphasize definite changes in incidence of intestinal protozoa and helminths. The incidence as given actually represents trends in the change of intestinal fauna and the progressive host-parasite relationships developing during a parasite-active interval in a young age segment of the village population.

There was considerable variation in the presence of protozoa and helminths in different individuals studied. In only a few cases were protozoans detected in the first stool and then regularly in subsequent fecal samples. Their presence was definitely erratic, with intervals of 2 to 3 and sometimes 4 to 6 weeks between their appearance on one date and the next. When present, there was an abundance of active trophozoites as well as cysts since a number of the infections apparently were young and in a propagative phase of establishment. Furthermore, freshly preserved stools of soft consistency favored the finding of both trophozoites and cysts.

A study of individual records of children in the two younger age groups, generally showed an increase in intestinal fauna quantitatively as well as qualitatively and there was greater regularity in the presence of parasites as the study progressed. Helminth eggs once found in an individual frequently appeared in subsequent samples examined.

In Group I (most of children 4 to 5 months old at start of study) the majority (62%) of the children were negative throughout the survey. Only one case of *Giardia lamblia* infection and one of *Trichomonas* were detected in the first lot of samples examined. Actually, the incidence of these two protozoans remained low for the first 5 weeks. There was a marked increase in protozoa during the sixth week (average age of subject, 5.5 months) and by the end of the survey 30 per cent of the group possessed *G. lamblia* and 9 per cent *T. hominis*.

Perhaps it is a matter of chance, but it should be noted that *G. lamblia* and *T. hominis* were not coexistent in the children studied. No protozoa were detected during the 16-week period in the three youngest members of Group I, all under 2 months of age at time the study was initiated.

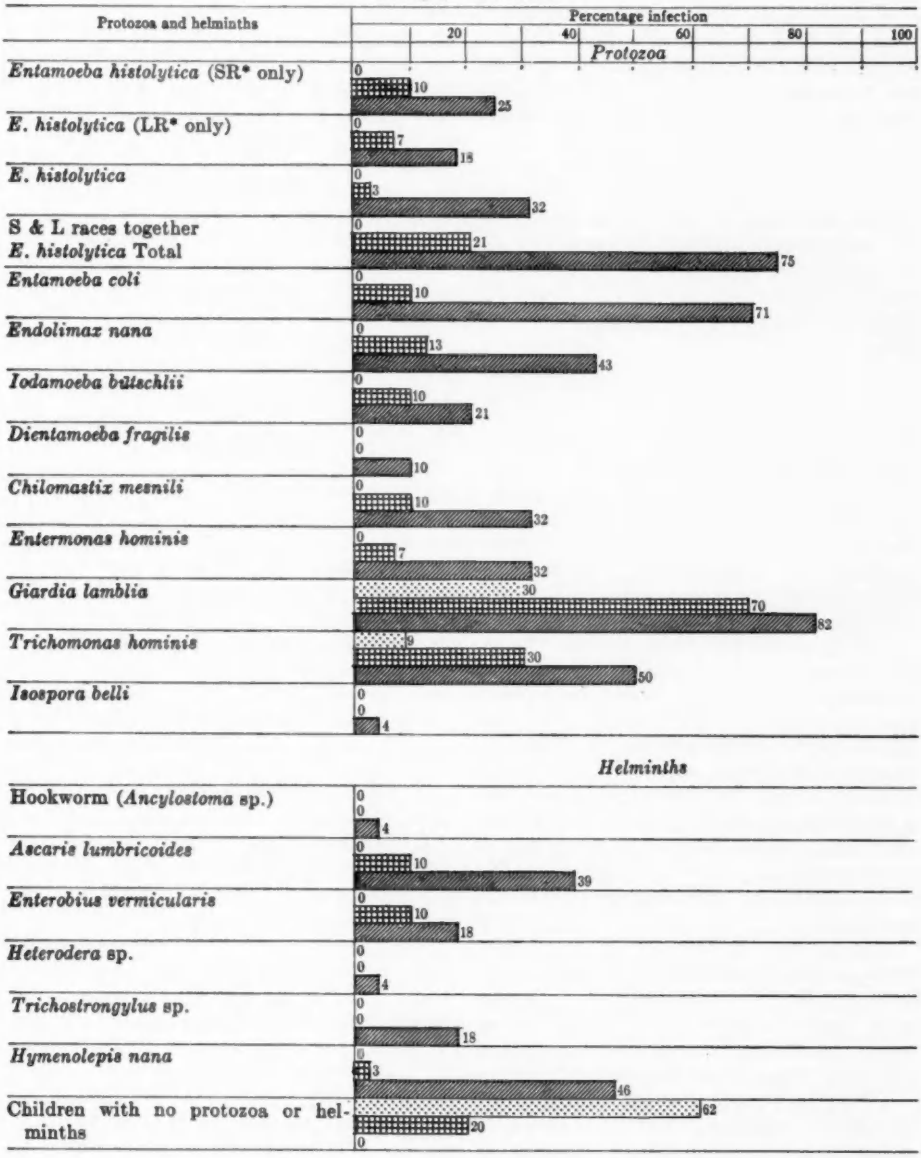
An evaluation of the data on individuals of the second group leaves no doubt that the period of 15 to 20 months is a very important one from the standpoint of establishment of intestinal fauna in children of the village. There was a rapid upswing in the incidence of the flagellate protozoans in children at 14 to 16 months. Eleven of the 30 children in Group II possessed *G. lamblia* and/or *T. hominis* on the first examination. *Chilomastix mesnili* appeared during the fourth week (child 17 months of age) and this organism, as well as *G. lamblia* and *T. hominis*, increased markedly in the group until there were incidences of 10, 70 and 30 per cent, respectively, at the termination of survey period. *Enteromonas hominis*, a flagellate found by us in more than one-third of the adult population, was also present (7%).

No amebas are reported for children in the 1-to-6-months age group (Group I). *Endolimax nana*, occurring in over half of adult Egyptians, was first detected in a child 18 months of age, *i.e.*, Group II. Four of 30 children in Group II possessed this ameba at termination of the survey. *Entamoeba histolytica* was detected in several of the older children of Group II 12 weeks after collection of the first fecal samples. There was one exception, a 2-year-old male, the oldest in the group, in which *E. histolytica* was found in a stool sample taken during the third week. Although earlier infections with this parasite have been noted in some of our surveys, data presented here indicate that children usually become susceptible to *E. histolytica* at 16 to 18 months, the rate of infection becoming progressively greater during the following 2 to 3 years. Chandler (1954) found over 50 per cent of children of Sindbis infected by the age of 4 years.

When first detected in Group II, either the small or the large race of *E. histolytica* was

TABLE 1

*Incidence (per cent) of intestinal protozoa and helminths occurring in groups of children examined weekly for 4 months*




* LR = large race *E. histolytica* with cysts consistently measuring more than 10 $\mu$; SR = cysts less than 10 $\mu$.

23 children 1 to 6 months of age at beginning of survey (Group I).
30 children 13 to 24 months of age at beginning of survey (Group II).
28 children 26 to 47 months of age at beginning of survey (Group III).

present in 5 of 6 cases, the sixth harbored both races. Although *Entamoeba coli* frequently shows a predominance in the adult Egyptian population (Lawless, *et al.*, 1956), the total rate for *E. histolytica* in children of Group II was twice that recorded for *E. coli*. *Iodamoeba bütschlii* appeared late in the study of Group II and in the three cases was in company with *E. histolytica*. *Dientamoeba fragilis* was absent.

Three helminths, *Ascaris*, *Enterobius* and *Hymenolepis nana*, made their debut in Group II. In each case these worms were coexistent with one to four species of protozoa, indicating, perhaps, that an effective multiparasite transmission condition existed. As a matter of fact, only 20 per cent of the group were negative for intestinal fauna.

In Group III the incidence for all parasites increased noticeably and additional species (*Dientamoeba fragilis*, *Isospora belli*, *Trichostrongylus*, and *Ancylostoma*) had made their entry. The presence of the eggs of *Heterodera* seem to indicate that the youngsters had reached the age when they were eating enough root vegetables for the eggs of this plant parasite to reach detectable levels. As a matter of fact none of these children escaped parasitism. The flagellates apparently flourish well in children 2 to 4 years of age, *G. lamblia* attaining a rate of 82 per cent. It seems likely that the incidence may have approached near to 100 per cent had more than single stool specimens been examined. *T. hominis* and *E. hominis*, frequently not even listed in general surveys, and *C. mesnili*, appeared in 50, 32 and 32 per cent, respectively, of the individuals of this age group. Apparently the human intestine at this stage also offers a more favorable habitat for the amebas since there was a total infection of 75 per cent with *E. histolytica* and 71 per cent with *E. coli*.

As in previous studies (Lawless, *et al.*, 1956; Wells and Blagg, 1956) of Egyptian adults, there was a predominance of the small race *E. histolytica*, *I. bütschlii* increased to 21 per cent and *D. fragilis*, not encountered in the lower age groups, occurred in 10 per cent of the stools.

Helminth parasites became well established and the eggs of *H. nana* and *Ascaris* were found in 46 and 39 per cent, respectively, of the stools examined, and *Trichostrongylus* and *Enterobius* each in 18 per cent. The latter figure is high and these results indicate that repeated perianal swabs might have shown an incidence approaching 100 per cent. Although we have previously recovered eggs of *Schistosoma mansoni* from children under the age of 3 and *S. haematobium* in slightly older children in the Sindbis area, none was detected in the present study. However, Weir, *et al.*, (1952) reported that 6 per cent of the Sindbis children between the ages of 1 and 4 passed eggs of *S. mansoni* with feces and approximately 28 per cent passed urine with eggs of *S. haematobium*. Infection rates for all helminths in children of 26 to 47 months age group were greater during the latter half of the survey, further supporting the supposition that this stage is one of active acquisition of intestinal fauna.

Surveys of the parasites of young children in different geographic areas seem to be limited. The present linear study on the intestinal fauna in children, although based upon a small sample of the population followed for a short period of time, definitely demonstrates the trend of parasite establishment in a community where the common parasites of man abound with little interference by medical care and/or improvement in sanitary conditions. In general, it seems likely that human beings reach a particular stage when their physiology and dietary habits allow, or favor, protozoa and parasite establishment. Some may hold that the increased activities of a young child, the consumption of greater quantities of contaminated foods, and the increased contact with sources of infection accompanying maturation are responsible for the presence of intestinal fauna at a certain age. This thesis cannot be accepted since even casual observations in villages, similar to that of Sindbis, convince one that a child is exposed to infection almost constantly beginning a few days after birth. Children are fed with dirty, contaminated hands, associate intimately with pets and domestic animals, the mucus membranes of the eyes, nose and mouth are attacked by numerous flies and at an early age they crawl on a dirt floor heavily contaminated by man and animals. Apparently a host-intestinal fauna tolerance or a coexistence relationship develops at a certain stage. Conditions which permit establishment of protozoa and helminths in the intestinal medium probably are dependent upon pH and other physiologic or biochemical characteristics relating to food intake and aging of the host.

Host-parasite relationships among peoples such

as the villagers of Sindbis leave an investigator baffled. It seems that infection of children and adults by so many parasites would result in even greater disability than that which is apparent in the population at large. As Weir (1952) and his group who worked in Sindbis have intimated, it seems likely that the village population lives in a symbiotic balance with its parasites.

SUMMARY

Children of three different age groups (1 to 6 months, 13 to 24 months and 26 to 47 months of age at beginning of survey) were studied weekly for their intestinal fauna for a period of 16 weeks. The intestinal fauna of the youngest group were limited quantitatively and qualitatively and only flagellate protozoans were detected. Stool examinations of children 13 to 24 months of age indicated that the period of 15 to 20 months was very important from the standpoint of general establishment of intestinal fauna in a child. Flagellates increased in number, amebas were detected and the eggs of *Ascaris*, *Enterobius* and *Hymenolepis nana* were passed with feces. At the age of 16 to 18 months *Entamoeba histolytica* became established, the rate of infection increasing rapidly the following 2 years. In the oldest age group protozoan and helminth populations increased markedly and none of the 28 children escaped invasion by intestinal fauna. A total of nine species of protozoa, plus both the large and small races of *E. histolytica* and six different helminths were found in the latter age group by the time this study terminated. Although the village of Sindbis lies in an endemic area, neither of the two schistosomes common to Egypt were found.

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# STUDIES ON TOXOPLASMOSIS

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## I. The Methylene-blue Dye Test in Normal Individuals

The Sabin-Feldman (1948) (1953) methylene-blue dye test, which is the most widely used test for the diagnosis of toxoplasmosis, provides the most sensitive serologic method for detection of *Toxoplasma* antibodies. Therefore, if a sudden change in antibody titer is noted in any given case, it is important to know how much of this change might be due to variation in the dye test, after ruling out experimental error. The following study was designed to measure this fluctuation.

### METHOD

Nineteen volunteers were selected at random from among the students at the University of California Medical Center. Blood was obtained from each volunteer at approximately the same hour on Day 1, 2, 4, 8 and 16 and the serum was separated and promptly stored at −10°C until tested. All sera drawn on any one date were examined simultaneously. In additional tests, all sera from each donor were also examined simultaneously.

*Dye test procedure.* Using separate pipettes in each tube, serial dilutions of serum were made so that 0.1 ml was progressively diluted. One part of freshly drawn, infected, mouse peritoneal fluid was added to 4 parts of accessory-factor serum. Of this mixture, 0.1 ml was added to the tubes, and they were incubated at a 37°C water bath for 1 hour. Following this a freshly prepared 1:5 mixture of saturated, alcoholic, methylene-blue dye and pH-11 buffer was added to the tubes which were then allowed to stand for 10 minutes to permit thorough staining. A drop of this mixture was subsequently placed on a slide and examined microscopically. One hundred toxoplasmas were counted in each tube of a serial dilution until the percentage of unstained organisms fell below 50. The last tube dilution containing 50 per cent or more of unstained toxoplasmas was reported as the dye-test titer.

To clarify any dye-test titer changes which are primarily a result of the 50 per cent end point method of reporting, we adopted Kass' (1954) method of reporting titers. Therefore, those tubes containing 50 to 59 per cent unstained toxoplasmas were labelled ±P, ("doubtful positive") and those with 60 per cent or more of unstained organisms were labelled P ("definite positive"). Tubes with 40 to 49 per cent unstained toxoplasmas were labelled ±N ("doubtful negative") and those with below 40 per cent were labelled N ("definite negative").

For each donor there was one dilution on which the majority of titer end points fell. This tube is called the "modal dilution," or mode.

### RESULTS

All 95 sera (100 %) fell within one 2-fold dilution of the respective modal dilutions. Also, in each individual case, the direction of the variation was constant.

Experimental results are shown in Table 1.

A duplicability study was also done. Eighteen Day-8 sera were arbitrarily selected and re-examined at a later date. Comparison of these results is shown in Table 2.

### CONCLUSION

Under the conditions outlined above it was demonstrated that the dye-test is reliable and that the titer in any normal individual remains constant within a 2-week period. In this group it did not vary by more than one 2-fold dilution, and then only in about 3 per cent of sera. This is within the variation inherent in the test, as shown by tests on a set of duplicate serum samples in which variation of one 2-fold dilution in about 17 per cent of cases was found.

Confusion in regard to dye-test "changes" is greatly reduced if the actual count of unstained organisms is reported along with the end-point titer.

[1] Supported by a Summer Medical Student Grant from the National Council to Combat Blindness, Inc., and a Summer Medical Student Grant from The American Cyanamid Corporation, Lederle Division.

TABLE 1

*Results of tests on 95 serum samples from 19 volunteers*

| Results of dye tests | Number of samples (among 95) | Per cent (of 95) |
|---|---|---|
| Fall on modal dilution | 75 | 79 |
| Fall one 2-fold dilution away from the mode: | | |
| Doubtful range (±P or ±N) | 17 | 18 |
| Definitely one 2-fold dilution from mode | 3 | 3 |
| Total | 20 | 21 |

TABLE 2

*Results of re-examination of 18 Day-8 Sera*

| Results of dye tests | Number of samples (among 18) | Per cent (of 18) |
|---|---|---|
| Agreement in same dilution titer | 12 | 67 |
| One 2-fold dilution away: | | |
| Doubtful range | 3 | 17 |
| Definitely one 2-fold dilution away | 3 | 17 |
| Total | 6 | 33 |

## II. Effect of the Toxoplasmin Skin Test on the Methylene-blue Dye Test

Some investigations have shown that certain bacterial products which are used to test dermal hypersensitivity may lead to an increase in specific antibodies, either in the total organism or in the tissue in the area of the intradermal test. Jawetz (1954) has pointed out that inoculation of skin-test antigens may stimulate or renew the complement-fixing antibody, or any other antibody that may be present.

One of the skin-test antigens used for surveys on the incidence of toxoplasmosis is produced from the peritoneal fluid exudate of a *Toxoplasma*-infected mouse. The antigen is produced by Eli Lilly Company[2], using methods described by Frenkel (1948).

The following study shows the effect of toxoplasmin on subsequent dye tests.

### METHOD

The technic of inoculation involved the injection of 0.1 ml of the skin-test antigen into the flexor surface of the left arm, and 0.1 ml of the control material into the corresponding surface of the right arm or into a skin area about 3 inches below the injection site of the antigen on the left arm. The areas of injection were examined after 48 hours. A minimum of 10 mm diameter of induration was considered skin-test positive.

Twenty-one volunteers were given a routine toxoplasmin skin test after withdrawal of an initial blood sample of 5 ml. The dye-test titer of this ample was examined together with four additional samples from each volunteer. The latter four sera were drawn on days 2, 4, 8, and 16 after the skin test, and processed as in Part I, above.

[2] The Lilly commercial skin antigen is 1:1000 dilutions of infected peritoneal fluid.

### RESULTS

*Skin tests.* Of the 21 volunteer subjects, four demonstrated a definitely positive skin test (Table 3).

*Dye tests.* Treating the negative and positive skin-tested individuals of this experiment as two separate groups the variations from the modal dilution were as shown in Table 4. Regardless of the way in which the data are grouped, 100 per cent of all serum samples were within one 2-fold dilution of the respective modal dilution.

Thirteen samples were checked for reliability

TABLE 3

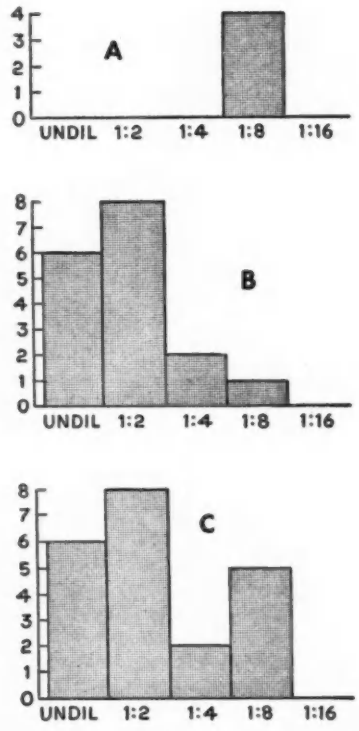
*Variations in dye-test titer after toxoplasmin intradermal tests*

| Results of dye tests | Dye tests on 83 serum samples from 17 volunteers with negative skin tests | | Dye tests on 20 serum samples from 4 volunteers with positive skin tests | |
|---|---|---|---|---|
| | No. | Per cent | No. | Per cent |
| Fall on modal dilution | 72 | 87 | 19 | 95 |
| One 2-fold dilution from mode: | | | | |
| In doubtful range | 7 | 8.5 | 1 | 5 |
| Definitely one 2-fold dilution from mode | 4 | 4.5 | 0 | 0 |
| Total | 11 | 13 | 1 | 5 |

TABLE 4

*Tests on 103 serum samples from 21 volunteers (positive and negative skin reactors)*

| Results of dye tests | No. | Per cent |
|---|---|---|
| Fall on modal dilutions | 91 | 88 |
| Fall one 2-fold dilution from mode: | | |
| In doubtful range | 8 | 8 |
| Definitely one 2-fold dilution from mode | 4 | 4 |
| Total | 12 | 12 |




Ordinates = dilutions; abscissae = nos. of persons.

A. Frequency distribution of dye-test titers in 4 positive-skin-tested subjects.

B. Frequency distribution of dye-test titers in 17 negative-skin-tested subjects.

C. Frequency distribution of dye-test titers of total (21) individuals.

of results. In each of the 13 sera, titer determinations were made on the same sample in two different tests. The variations were within experimental limits.

Chart I shows the findings on negatives and positives grouped together (C) in a distribution study of the end-point titers. In each case the 21 modal dilution titers were distributed between undiluted and 1:8. When considered separately, (A) indicates that all four positives showed a 1:8 titer, which is well above the mean titer (1:2) of the negatives (B).

The incidence of individuals with titers above 1:16 is very much lower in this small sample than that found by Feldman (1953). This difference could be due to the older age group in Feldman's Study and also to the variation due to geographical area surveyed.

### CONCLUSIONS

1. The adminstration of toxoplasmin did not cause an appreciable change in the dye-test titer of individuals with either positive or negative skin tests up to a period of 2 weeks post-injection. The single 2-fold dilution variation which occurred was within the range of experimental error.

2. None of the volunteers in this group demonstrated a titer higher than 1:8. This is well below the titers in a normal population average as found by Feldman (1953) in other geographical areas.

## III. A Chemotherapy of Experimental Mouse Toxoplasmosis

This group of experiments was performed to re-evaluate the effects of certain drugs which have been reported, or suspected, to be effective against *Toxoplasma*. The drugs[3] are hydrochloroquine sulfate[4] (HCS) (Eyles and Jones, 1947); chloroquine diphosphate[5] (CPD) (Eyles and Coleman, 1953, 1955a, 1955b); methoxyquinidine phosphate[6] (MQP) (Eyles and Coleman, 1953, 1955a, 1955b). *Toxoplasma* of the RH strain, isolated by Sabin in 1941 and maintained in these laboratories by constant

[3] Obtained through the courtesy of the Department of Medical Research, Winthrop-Stearns, Inc.

[4] Plaquenil®.

[5] Aralen®.

[6] Primaquine®.

mouse passage sinc 1947, was used in all the studies.

Four experiments were performed to establish the effectiveness of various drugs in prolonging the life of animals infected with *Toxoplasma*. In each case the drugs were given immediately after the infecting inoculum.

EXPERIMENT I

This was planned to determine the toxicity of HCS, CDP and MQP when given subcutaneously once each day, and the therapeutic value of one-half this dose (Weil, 1952).

*Method*

To determine toxicity three different dosages of each drug, based on clinically recommended amounts, were used. The drugs were given to 20-gm laboratory mice on a weight-per-kg basis. Once the toxic level was determined, a dose exactly one-half the toxic amount was tried as a chemotherapeutic agent in mice. These dosages were:

| | | |
|---|---|---|
| HCS | 5 mg/day | (250 mg/kg/day) |
| CDP | 1.8 mg/day | (90 mg/kg/day) |
| MPQ | 0.75 mg/day | (37.5 mg/kg/day) |

Three mice in each of four groups were infected intraperitoneally with 50,000 toxoplasmas, and then treated immediately with the above dosages by daily, subcutaneous administration. One group was left as a control.

RESULTS

There was no significant difference in survival time between treated mice and controls.

We also found that more than four subcutaneous injections of HCS and MPQ at the same site produced necrosis and sloughing of the overlying skin. The concentrations of the drugs (in normal saline) were: HCS, 10%; MPQ, 0.3%.

EXPERIMENT II

This study was planned because of the necrotic effects following subcutaneous administration of the drug solutions. Oral administration (the medications being homogenized and granulated in the food) of the same dosage, based on a daily 4-gm food intake, was tried. It was felt that a smaller inoculum was desirable; therefore, an infective dose of 5,000 toxoplasmas each was given intraperitoneally to three mice in each of four groups, as in Experiment I.

TABLE 5

*Survival of mice after infection and treatment*

| Drug | Gram % in diet | Average days survival |
|---|---|---|
| HCS | 0.125 | 6.4 |
| CDP | 0.045 | 6.3 |
| MQP | 0.015 | 6.2 |
| None (controls) | — | 6.4 |

*Results*

The results of Experiment II are shown in Table 5.

*Conclusion*

Oral administration of the drugs tested showed no significant prolongation of survival time after infection with 5000 toxoplasmas.

EXPERIMENT III

An experiment was planned to compare the antitoxoplasmic activity of sulfapyrazine and sulfadiazine alone and in combination with pyrimethamine[7] (PMA) (Summers, 1953; Eyles and Jones, 1955; Eyles and Coleman, 1955a, 1955b).

*Method*

Inasmuch as this was considered to be a pilot study for a larger experimental group if the results warranted, only three mice were used in each group. Five thousand toxoplasmas were inoculated intraperitoneally. The drugs were homogenized and granulated into the food and the animals were allowed free access to the food and water. Fourteen days after inoculation of the organisms the surviving animals were returned to a normal diet for 14 more days.

Upon the death of a mouse microscopic examination of the peritoneal fluid was immediately made, for evidence of infection by *Toxoplasma*. If no toxoplasmas were found by direct smears, the brains were inoculated into healthy mice. At the end of the experiment the surviving mice were sacrificed. Each brain was subinoculated intraperitoneally into two fresh mice. Subsequent infection with toxoplasmas was determined by microscopic examination of the peritoneal fluid.

[7] Daraprim® manufactured by Burroughs, Wellcome & Co.

TABLE 6

*Survival and drug cures with the various tested drugs*

| Drugs | Gram-% in diet | Animals | | Deaths on days postinfection | | | | | | | | | | | | | Fraction living at end of 4 weeks | Subinoculated animals dying with toxoplasmosis | Original animals with complete cure | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Group* | No. | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | | | Ratio | % |
| Sulfapyrazine | 0.03 | I | 10 | | | | | | | | | 1 | | | 1 | 1 | | 6/10 | 3 | 3/10 | 30 |
| | | II | 9 | | | | | | 1† | | | | | | | | | 8/9 | 0 | 9/9 | 100 |
| Sulfapyrazine and PMA | 0.0075 | I | 10 | | | | | | | | | | | | | | | 10/10 | 0 | 10/10 | 100 |
| | 0.005 | II | 10 | | | 1† | | | | | | | | | | | | 9/10 | 0 | 9/9 | 100 |
| Sulfadiazine | 0.250 | I | 11 | | | 1† | | 2† | | | | | | | | | 1 | 7/11 | 1 | 6/8 | 75 |
| | | II | 10 | | | | | | | | | | | | | | | 10/10 | 0 | 10/10 | 100 |
| Sulfadiazine and PMA | 0.06 | I | 10 | | | | | | | | | | | | | | | 10/10 | 0 | 10/10 | 100 |
| | 0.005 | II | 10 | | | 1† | | | | | | | | | | | | 9/10 | 0 | 9/9 | 100 |
| S-S | 0.250 | I | 9 | | | | | | | | | | | | | | | 9/9 | 3 | 6/9 | 66 |
| | 0.250 | II | 10 | | | | | | | | | 1 | | | | | | 9/10 | 2 | 7/10 | 70 |
| S-S 0.06 (plus PMA 0.005) | 0.065 | I | 10 | | | | | | | | | | | | | | 1 | 9/10 | 3 | 6/10 | 60 |
| | 0.065 | II | 11 | | | | | | | | | | | | | | | 11/11 | 0 | 11/11 | 100 |
| Controls | — | | 3 | | | | 3 | | | | | | | | | | | 0/3 | 3 | 0/3 | 0 |

* Group I, 14-day medication; Group II, 28-day medication.

† Animal died of fight wounds from other mice. Toxoplasmas were not demonstrated.

*Results*

The results of this study may be listed briefly as follows:

1. All controls died within the first 5 days.
2. No animals receiving combined therapy died during the 28 days of observation.
3. No deaths occurred in the first 14 days.
4. One sulfapyrazine-treated mouse died on the 27th day post-infection; three PMA-treated mice died on the 17th, 21st and 27th post-infection days, respectively.

*Conclusions*

Based on the data obtained in Experiment III the following conclusions seem warranted:

1. PMA alone in the dosage tested (0.025 gm % in diet) was not curative but suppressed the toxoplasmas as long as it was given.
2. Sulfadiazine alone (0.500 gm % in diet) cured two mice and suppressed the infection in one. Sulfapyrazine alone (0.1030 gm % in diet) cured one and arrested the disease in two.
3. All animals receiving sulfadiazine and PMA, or sulfapyrazine and PMA, in dosages noted above were apparently cured.

EXPERIMENT IV

This experiment was performed to compare the antitoxoplasmic activity of the drugs used in Experiment III with another antitoxoplasmic drug, Solu-Supranol[8] (S-S), a mixture of equal weights of sulfamylon and sulfamerazine, which has been used with reported success in Germany (Frank, 1955).

*Method*

This experiment was performed in the same manner as Experiment III, with two modifications:

1. The infection was with 20,000 toxoplasmas intraperitoneally.
2. The animals used were divided into two groups of about ten each for each drug or combination of drugs.

All Group I animals were kept on the drug diet for 14 days, followed by 14 days on normal diet. All Group II animals were kept on the drug diet for 28 days.

All animals which died were autopsied and subinoculations were made into normal mice. Twenty-eight days after infection the surviving animals were sacrificed and subinoculations made as described above.

*Results*

The results of Experiment IV are shown in Table 6. The animals on drugs for 28 days tended to exhibit a higher percentage of total cure than those on a drug for 14 days.

The sulfonamide combinations with PMA showed higher percentages of total cure than those without PMA, even though the actual amount of drug in the diet was only one-fifth that amount used without PMA.

*Conclusions*

1. In general, the sulfonamide drugs tested alone, or in combination with PMA, showed definite curative properties against toxoplasmosis, though the cure rate when used with PMA was equal or higher in all experiments.
2. There is evidence of synergism between the sulfonamide drugs tested and PMA (actual synergistic rate was not determined).
3. There is a slightly greater percentage cure rate with 28-day drug therapy than with 14-day therapy.
4. Sulfapyrazine seems to be approximately as effective against toxoplasmosis as sulfadiazine and S-S.

SUMMARY

*Toxoplasma-methylene-blue dye-test variation.* Five serum samples from 20 subjects were tested for dye-test titer, the samples being drawn over a period of 16 days. This was repeated in another 20 subjects, each of whom was given one skin-test dose of toxoplasmin before beginning the serum-withdrawal series.

In both series the dye-test titers remained remarkably constant for each individual, and did not change after the preceding injection of toxoplasmin. This work also established the repeatability of the serologic dye-test determination.

*Chemotherapy of toxoplasmosis.* Several antimalarial drugs were tested for effectiveness in prolonging the life of mice after infection with a highly lethal dose of *Toxoplasma* (RH strain). Oral or subcutaneous drug administrations were employed. None of these (Plaquenil, Aralen, and Primaquine) showed any effect.

[8] This drug is not registered in the United States, but is termed such in Frank's handbook (1955).

Various sulfonamide compounds, both alone and in combination with pyrimethamine, were tested as drug cures. The most effective combinations were sulfapyrazine-pyrimethamine, and sulfadiazine-pyrimethamine mixtures, substantiating the work of Eyles and others. Another double-sulfa mixture (sulfamylon and sulfamerazine) was tested and also found to be very effective as a drug cure in acute toxoplasmosis, especially when used with pyrimethamine. These experiments also demonstrated the synergism existing between certain sulfonamides and pyrimethamine against toxoplasmosis in mice.

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# FACTORS INFLUENCING TRANSMISSION OF JAPANESE B ENCEPHALITIS VIRUS BY A COLONIZED STRAIN OF *CULEX TRITAENIORHYNCHUS* GILES, FROM INFECTED PIGS AND CHICKS TO SUSCEPTIBLE PIGS AND BIRDS

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## INTRODUCTION

Studies of encephalitis in Japan have shown *Culex tritaeniorhynchus* to be the mosquito species most commonly *infected* in nature by Japanese encephalitis (JE) virus (Mitamura *et al.*, 1938; Hammon, 1949a; Annual Professional Report, 406th Medical General Laboratory (MGL), 1950–56). Despite numerous recoveries of JE virus from *C. tritaeniorhynchus*, conclusive evidence that this mosquito actually *transmits* the virus to *natural hosts* has been lacking because of difficulties in colonizing the mosquito. Evidence that this species of mosquito can transmit JE virus has been afforded by several groups of workers. Mitamura *et al.*, (1938) transmitted virus to mice through bites of naturally infected *C. tritaeniorhynchus*, collected from an epidemic area. Hammon *et al.*, (1949b) collected *C. tritaeniorhynchus* in nature as larvae and reared them to adults in the laboratory. Eight days after infection of these mosquitoes by feeding them JE virus (Nakayama strain) as infected mouse-brain suspension on cotton, the mosquitoes transmitted virus to one of three mice. The titer of virus in the brain was not reported, but presumably was high since a 3 per cent suspension of brain in defibrinated rabbit's blood was employed to infect the mosquitoes. Hammon *et al.*, (1949b) recognized that transmission of JE virus by *C. tritaeniorhynchus* to unnatural hosts such as mice, after feeding the mosquitoes virus suspensions of high titer, did not necessarily prove that *C. tritaeniorhynchus* was a vector of JE virus in nature. Hale *et al.*, (1957) demonstrated transmission of virus to chicks in three instances after allowing laboratory-reared Malaysian *C. tritaeniorhynchus* to feed on infected mouse brain in defibrinated rabbit blood.

When a colony of these mosquitoes was recently established by Newson *et al.*, (1956), experiments were undertaken to learn whether *C. tritaeniorhynchus* could transmit JE virus among natural hosts such as birds and pigs, and to study the vector efficiency of *C. tritaeniorhynchus* by investigating the influence of host blood virus titer on infection and transmission rates of the mosquito.

The roles of birds and domestic pigs in the ecology of Japanese encephalitis have been studied intensively by the Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory. The results of studies during the summers of 1952–56 will be presented in detail in manuscripts now in preparation. In brief, they revealed several factors that account for the importance of these animals in the ecology of JE virus near Tokyo, Japan: 1) birds and pigs are common animals in the Kanto plain around Tokyo and the rapid yearly population turnovers maintain a large population susceptible to JE virus infection; 2) wild birds and domestic pigs become infected by JE virus in nature as evidenced by the development of antibodies and by the recovery of virus from blood; 3) experimentally-inoculated birds and pigs become viremic for as long as 4 days; 4) hundreds to thousands of *C. tritaeniorhynchus* mosquitoes have been collected in one night from bird- or pig-baited traps, thus suggesting the potentially high effectiveness of viremic birds and pigs as sources of infection for vector mosquitoes. It remained necessary to prove, however, that birds and pigs could actually infect mosquitoes and that these mosquitoes were, in turn, infective for other birds and pigs.

In the experiments reported, domestic pigs and baby chicks were employed as sources of virus

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for the infection of *C. tritaeniorhynchus*; subsequently, these mosquitoes transmitted virus to a pig, Black-crowned Night Herons, egrets and chicks. Since both pigs and wild birds, such as herons and egrets, are hosts of JE virus in nature, the demonstration that *C. tritaeniorhynchus* can effect a complete cycle of this virus from natural host to natural host furnishes further evidence that *C. tritaeniorhynchus* is a natural vector of JE virus in Japan.

## MATERIALS AND METHODS

*Mosquitoes.* Adult female *C. tritaeniorhynchus* were reared from egg rafts of the 12th to 17th generation of mosquitoes from a colony established during the summer of 1956 by Newson *et al.*, (1956) in the Department of Entomology, 406th Medical General Laboratory. Both uninfected and infected adult female mosquitoes were kept at 23–25°C at a relative humidity of 80–90 per cent and were subjected only to the approximate 11 to 13.5 hours of daylight prevalent between January and May, 1957 and not to the extended period of light found essential for breeding (Newson *et al.*, 1956).

*Virus.* Japanese encephalitis virus, strain M5/596, isolated originally from a pool of 14 *C. tritaeniorhynchus* caught at the Sagiyama heronry near Tokyo in August, 1955, was employed throughout the experiments. It was stored with dry ice as a 1 per cent suspension of infected brains of the fourth mouse passage in diluent consisting of four parts of normal rabbit serum (NRS), free of neutralizing antibody to JE virus, and six parts of normal saline. For each experiment, a separate ampule was used and the titers of infectious virus at the beginning and end of these experiments were $10^{-8.4}$ and $10^{-8.5}$ $LD_{50}$/0.04 gm of brain by intracranial inoculation of 3 to 4-week-old weanling albino mice.

*Infection of mosquitoes.* After emergence from pupae, young female mosquitoes were placed in cylindrical jars, 8.25 inches in diameter and 8.25 inches in height. The jar bottoms were covered with a piece of wet cotton. For the first 3 days after emergence the mosquitoes fed on a ball of cotton soaked in a 2 per cent sugar solution suspended inside the jar. On the fourth and fifth days, the sugar solution was removed, and on the sixth and seventh days, the pad of wet cotton was also removed. These deprivations of food and water stimulated the mosquitoes to bite pigs and chicks.

To infect mosquitoes from pigs, a 5-month-old pig from the Kanto area, No. 278 and a 3-month-old pig from Hokkaido, Japan, No. 257, were inoculated subcutaneously in the ear with 160,000 weanling-mouse-$LD_{50}$ of JE virus in a volume of 0.2 ml. Both pigs were shown by neutralization test to be free of antibody to JE virus at the time of inoculation. On four successive evenings, the pigs were immobilized for one-half hour and *C. tritaeniorhynchus* were exposed to each pig's shaved abdomen. A different group of mosquitoes was exposed each evening. Prior to each exposure to mosquitoes, 5 ml of blood was drawn from each pig, promptly diluted decimally in 10 per cent NRS in saline, and the titer of virus determined by intracranial inoculation into weanling mice.

To infect mosquitoes from chicks, lots of 200–500 mosquitoes were placed for 16 hours in separate jars, each containing a viremic chick immobilized by the taping of wings and legs. Chicks with JE virus in the blood were prepared by inoculating 2-day-old white leghorns subcutaneously in the right breast with 160,000 mouse-$LD_{50}$ of virus in 0.2 ml volume. Thirty hours after inoculation of virus, each chick was bled (1 ml) from the jugular vein and exposed to mosquitoes. This blood, and blood obtained immediately after 16 hours of exposure to mosquitoes, were titrated in weanling mice.

*Transmission of virus by mosquitoes.* After feeding on a viremic pig or chick, mosquitoes judged macroscopically to have ingested blood were transferred in groups of 20 to lamp chimneys placed on moist cellucotton, and covered at each end by cloth mesh (holes 2 x 2 mm in size = 19 mesh) with a slice of apple on the top screen to provide food. To determine the ability of mosquitoes to transmit virus after their exposure to a viremic pig, they were allowed to bite a susceptible pig, Black-crowned Night Herons (BCNH), egrets and 2 to 7-day-old chicks. These mosquitoes were deprived of their usual food (a slice of apple) for 2 days and water for 1 day before exposure to the susceptible animal. Pigs were exposed by holding the screened end of a lamp chimney containing mosquitoes against the skin for 30 minutes. Herons and egrets were immobilized for one-half hour in a cage 18 in. x 18 in. x 18 in. and chicks were exposed by strapping them for 3 hours to the top screen of the lamp chimney. The susceptible pig, No. 293, was a 3-month-old Yorkshire from Hokkaido, and the susceptible

BCNH were young adults captured when they were nestlings at the Shinhama heronry during the summer of 1956 and kept in the aviary at the 406th Medical General Laboratory for 8 months prior to use in these experiments. Plumed and Great Egrets (PE and GE) were captured at the Shinhama heronry as adults and kept in the aviary for 3 weeks prior to use. Neutralization tests, as outlined by Paul (1944), were performed on the sera of pigs, wild birds and chicks employed in transmission experiments. Those without demonstrable neutralizing antibody in unheated, undiluted plasma (LNI less than 1.0) were considered to be susceptible to JE virus.

Following their exposure to infected mosquitoes, susceptible animals were kept in a room separate from that of the infected mosquitoes. Pre- and postexposure plasmas from pigs, herons, egrets and chicks were tested for antibody by the hemagglutination-inhibition (HI) test of heated (56°C, 30 minutes), acetone-extracted plasma (Sabin and Buescher, 1950; Annual Professional Report, 406th MGL, 1953) and by the virus-dilution-neutralization test employing unheated, undiluted heparinized plasma (Paul, 1944). For the HI test, the first plasma dilution was 1:10, and the rise in antibody from less than 1:10 to 1:20 or greater by the twenty-first day after exposure to infected mosquitoes was considered as evidence of infection of the animal by JE virus and, therefore, as evidence of transmission of virus by the mosquitoes. Neutralization indices of greater than log 1.7 were taken to indicate presence of neutralizing antibody and of less than 1.0 as absence of antibody.

For quantitative data in transmission experiments, mosquitoes were exposed individually to susceptible chicks.

*Recovery of virus from mosquitoes.* Approximately 12 hours following exposure of mosquitoes to susceptible chicks for transmission studies, single or small groups of mosquitoes were transferred to test tubes. Usually, only the mosquitoes visibly engorged with blood were chosen and the nonengorged mosquitoes were left for re-exposure to other susceptible animals. The mosquitoes were immobilized by immersion of the test tube in an ice bath for 10 minutes. After transfer to a Ten Broeck grinder, lots of less than 25 mosquitoes were triturated in 0.75 ml of diluent (10 per cent NRS in saline with penicillin 500 units/ml and streptomycin 250/ml). For more than 25 mosquitoes, 0.04 ml of diluent was used per mosquito. Suspensions containing more than 10 mosquitoes were centrifuged at 400 G for 10 minutes at 4°C and inoculated (0.01 ml intracranially and 0.02 ml subcutaneously) into 1 to 4-day-old mice from two litters. The mice were observed daily for 14 days for signs of illness or death from JE virus.

In several of the initial experiments, to establish unequivocally the presence of JE virus in the mosquitoes, brains from sick mice were tested by complement-fixation test (Havens *et al.*, 1943) against 8 units of guinea pig JE antibody. This antigen fixed complement with JE antibody and not with guinea pig antibodies to St. Louis encephalitis and Western equine encephalitis viruses. In most experiments, the clinical course with culmination in death of mice between the fourth and tenth days after inoculation was deemed sufficient evidence of JE virus in the mosquitoes under test.

*Determination of titer of virus in an individual mosquito.* All calculations of virus titer in weanling mice were based on the method of Reed and Muench (1938). Each mosquito was triturated in 0.75 ml of diluent and each mouse inoculated with 0.04 ml or $\frac{1}{19}$ of the mosquito suspension. To approximate the number of weanling-mouse-intracranial $LD_{50}$ present in the whole mosquito, the experimental titer per 0.04 ml was, therefore, increased 19-fold.

## RESULTS

*Transmission of JE virus from pig to pig, BCNH, egret and chick.* Infection of colonized *C. tritaeniorhynchus* by JE virus caused neither detectable signs of illness nor death of the mosquito. For demonstration of infection it was necessary either to recover infectious JE virus from the mosquito by trituration and inoculation into mice or to transmit the virus to susceptible animals.

To infect *C. tritaeniorhynchus* with JE virus, groups of 300–1,200 mosquitoes were exposed to two viremic pigs (Table 1). Virus was present at low levels in each pig's blood following subcutaneous inoculation of 160,000 mouse-$LD_{50}$ of virus. On the second and third days after inoculation, virus reached maximal blood levels of log 2.5 to 3.3 mouse-$LD_{50}$/0.04 ml and, by the fourth day, was detectable at a very low blood level in both pigs.

The mosquitoes from Lot A (Table 1) fed on pig No. 278 one day after inoculation of virus

TABLE 1

*Infection of* Culex tritaeniorhynchus *by exposure to pigs circulating JE virus*

| Pig No. | Days after inoculation of virus | Virus in pig blood | | No. of mosquitoes engorging on pig / No. exposed | Mosquito lot designation |
|---|---|---|---|---|---|
| | | Titers* in weanling mice | Suckling mouse mortality | | |
| 278 | 1 | <0.0 (1/4)† | 10/10† | 27/500 | A |
| | 2 | 2.5 | 9/9 | 50/700 | B |
| | 3 | 2.3 | 13/13 | 55/300 | C |
| | 4 | <0.0 (0/4) | 3/10 | 55/350 | Not tested |
| 257 | 1 | 0.4 | 8/8 | 207/800 | Not tested |
| | 2 | 3.3 | 3/3 | 75/1200 | D |
| | 3 | 3.3 | 5/5 | 347/1000 | E |
| | 4 | <0.0 (2/6) | 7/8 | 289/700 | Not tested |

* Negative logarithm of weanling-mouse-$LD_{50}$/0.04 ml of blood.

† No. of mice dead/no. inoculated.

when the blood virus titer was less than log 0. After being in the insectary for 20 days, these mosquitoes were placed individually in separate lamp chimneys and exposed to susceptible chicks. None of the 30 chicks exposed to 21 mosquitoes on 2 successive nights developed antibody, and no virus was recovered from the mosquitoes of Lot A upon testing in suckling mice.

Sixteen mosquitoes from Lot B were kept 18 days after feeding on viremic pig No. 278, and then were exposed to a susceptible 3-month-old Yorkshire pig (No. 293). Eight of the 16 mosquitoes ingested blood from pig No. 293 and seven of these eight subsequently were shown to contain virus, ranging in titer from log 4.8 to log 5.1 (Table 2). Following the bites of these mosquitoes pig No. 293 was infected with JE virus, as evidenced by the development of hemagglutination-inhibition (HI) and neutralizing antibodies in the plasma (Table 2).

Mosquitoes from Lot C were also kept 18 days after ingestion of viremic blood. Four mosquitoes from this group were exposed to two BCNH. Three mosquitoes ingested blood and were shown to be both infected and infective for the BCNH, since antibodies promptly developed in the plasmas of the two BCNH (Table 2). Of 33 mosquitoes exposed individually to 33 chicks, eight mosquitoes engorged and seven were infected with JE virus (Table 2). Six transmissions of virus occurred as demonstrated by the development of HI antibody in six chicks.

Twenty days after ingesting viremic pig blood, three mosquitoes from Lots D and E were exposed separately to two adult PE and to one adult GE. All of the egrets became viremic and the 2 PE developed JE virus antibodies (Table 2).

*Transmission of JE virus from chick to chick by* C. tritaeniorhynchus. To test transmission potential of an infected mosquito for chicks, several difficulties had to be overcome. 1) The chick had to be susceptible to JE virus infection (approximately 10% of chicks were immune since they had maternal antibody in unheated, undiluted plasma by neutralization test with JE virus). 2) The chick, after exposure to an infected mosquito, had to survive 3 weeks to provide a second serum specimen for demonstration of antibody development indicating infection of the chick by the mosquito. 3) The mosquito had to survive the period of starvation prior to exposure to the susceptible animal. 4) Only mosquitoes having ingested blood (gross examination) were tested for the presence of virus. These four factors in transmission attempts materially reduced the total numbers of mosquitoes tested for transmission. However, transmissions from chick to chick did occur on 26 occasions (Table 3, Lots II–V).

*The effect of blood-virus titer upon infection and transmission rates of* Culex tritaeniorhynchus. *C. tritaeniorhynchus* were easily infected by ingestion of low concentrations of virus in both chick and pig blood (Table 3). If *C. tritaeniorhynchus* ingested approximately 0.002 ml of blood (Schneider and Fowler, unpublished data), each mosquito in Lot I might then contain approximately 100 $LD_{50}$ of virus immediately after feeding and, in Lots II and III, the concentra-

TABLE 2

*Transmission of JE virus from pig to pig, Black-crowned Night Herons, egrets and chicks*

| Mosq. lot designation | Extrinsic incubation period (*days*) | Exposure of susceptible animals to pig infected mosquitoes: Susceptible animal | Mosquitoes engorged / No. exposed | Virus in engorged mosquitoes tested individually: No. infected | Titer: Geom. mean (range) | Mosquito transmission to susceptible animals: Viremia† (days) 1 | 2 | 3 | Test* | Antibody response: Pre-bleed | 3rd week | 7th week |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B | 18 | Pig #293 | 8/16 | 7 | 4.9(4.8–5.1) | Not tested | | | HI | <10 | 80 | NT |
| | | | | | | | | | Neut | 0.2 | 2.4 | NT |
| | 18 | BCNH #27 | 1/2 | 1 | 5.6 | | | | HI | <10 | 160 | NT |
| | | | | | | | | | Neut | 0.1 | 2.0 | NT |
| C | 18 | BCNH #24 | 2/2 | 2 | 5.1(4.8–5.4) | Not tested | | | HI | <10 | 320 | NT |
| | | | | | | | | | Neut | 0.1 | <1.4 | NT |
| | 18 | Chicks #1–33 | 8/33 | 7 | 4.8(3.8–5.8) | | | | HI | Six chicks developed HI antibody by the 14th day | | |
| | 20 | Plumed Egret #2249 | 1/1 | 1 | 4.7 | <0.0 | 0.5 | 1.8 | HI | <10 | NT | 80 |
| | | | | | | | | | Neut | 0.8 | NT | 3.8 |
| D & E | 20 | Plumed Egret #2250 | 1/1 | 1 | 4.9 | <0.0 | 1.6 | 1.5 | HI | <10 | NT | 40 |
| | | | | | | | | | Neut | 0.6 | NT | 2.8 |
| | 20 | Great Egret #2251 | 1/1 | 1 | 5.3 | <0.0 | 1.8 | 1.2 | HI | <10 | NT | Dead |
| | | | | | | | | | Neut | 0.7 | NT | Dead |

* Results of hemagglutination inhibition (HI) antibody tests are given as the reciprocals of the serum titers, and of neutralization tests, as logarithms of the neutralization indices.

† Viremia is expressed as the negative logarithm of the $LD_{50}$ of virus/0.04 ml of blood upon inoculation intracranially into weanling mice.

tion of virus per mosquito would be approximately 12.5 and 1 to 2 $LD_{50}$, respectively. During the extrinsic incubation period of virus in the mosquito, virus was often not recovered upon trituration of mosquitoes and inoculation into mice. Between 5 and 34 days after feeding on a viremic animal, the percentage of infected mosquitoes was related to the original concentration of virus in the blood of the host animal. For example, when the titer of virus in the animal was in the range 225 to 2,000 $LD_{50}$/0.04 ml (Lots I and II, Table 3), 93 to 94 per cent of mosquitoes were found to be infected; at a blood level of 16 to 32 $LD_{50}$/0.04 ml, the range was 47 to 58 per cent (Lots III, IV); at a blood level of 3 $LD_{50}$/0.04 ml (Lots V and VI), only 22 per cent of the mosquitoes were infected after feeding on either a viremic chick or pig. The higher overall transmission rates in mosquito Lots I and II, 92 per cent and forty six per cent, respectively, as compared with Lots III, IV and VI, 29 per cent, 24 per cent and 17 per cent, respectively, can be attributed in part to the greater opportunity for transmission of virus when greater percentages of the mosquitoes in a group were initially infected. If only the percentage of *infected* mosquitoes that transmitted is considered (Table 3) it is apparent that the transmission rates of Lots I and II (92 per cent and 48%; ave. 70%) averaged to about the same value as Lots III, IV and VI (57%, 45% and 83%; ave. 61%). Thus, about 65 per cent of infected, colonized *C. tritaeniorhynchus* transmitted virus under the conditions of these experiments.

The titers of JE virus in individual *C. tritaeniorhynchus* transmitting virus to susceptible chicks and those failing to transmit are illustrated in Table 4. It can be seen that the geometric mean titers were similar in both groups.

*Temporal relationship between development of*

TABLE 3

*Influence of the infecting virus dose on frequency of mosquito infection and transmission*

| Mosq. lot No. | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| Host | Pig | Chick | Chick | Chick | Chick | Pig |
| Geom. mean titer* of virus in blood | 3.3 | 2.4 | 1.5 | 1.2 | 0.4 | 0.4 |
| Range of titer* | 3.3 | 2.2–2.6 | 1.4–1.6 | 1.0–1.4 | 0.2–0.6 | 0.4 |
| **Infection†** | | | | | | |
| Extrinsic incubation period (*days*) | | | | | | |
| 1 | NT‡ | 9/9 | 6/11 | 2/8 | 0/2 | NT |
| 2–4 | NT | NT | 0/6 | 1/6 | 0/6 | NT |
| 5–9 | NT | 7/10 | 6/20 | 9/30 | 3/10 | NT |
| 10–14 | 12/14 | 21/21 | 18/26 | 19/40 | 4/24 | 4/15 |
| 15–19 | 5/5 | 18/19 | 12/18 | 16/31 | 1/3 | 6/25 |
| 20–24 | 12/13 | 2/2 | 6/13 | 14/21 | NT | 3/18 |
| 25–29 | 9/9 | 10/10 | 8/9 | 4/9 | NT | 4/18 |
| 30–34 | NT | 3/3 | NT | 1/2 | NT | NT |
| Total 5–34 days | 38/41 (93%) | 61/65 (94%) | 50/86 (58%) | 63/133 (47%) | 8/37 (22%) | 17/76 (22%) |
| **Transmission§** | | | | | | |
| 10–14 | NT | 0/6 | 0/2 | 0/2 | NT | NT |
| 15–19 | 2/2 | 5/12 | 1/4 | 2/13 | NT | 0/4 |
| 20–24 | 3/4 | NT | 1/6 | 3/13 | NT | 1/8 |
| 25–29 | 7/7 | 8/9 | 2/2 | 3/8 | NT | 4/17 |
| 30–34 | NT | 0/1 | NT | 1/1 | NT | NT |
| Total 10–34 days | 12/13 (92%) | 13/28 (46%) | 4/14 (29%) | 9/37 (24%) | NT | 5/29 (17%) |
| **Transmission by *infected* mosquitoes‖** | | | | | | |
| Total 10–34 days | 12/13 (92%) | 13/27 (48%) | 4/7 (57%) | 9/20 (45%) | NT | 5/6 (83%) |

* Titer is expressed as the negative logarithm of weanling-mouse-$LD_{50}$/0.04 ml of blood. Pigs were exposed for 0.5 hour to mosquitoes. Consequently, only one determination of blood virus titer was deemed necessary. Chicks were exposed to mosquitoes 30 hours after inoculation of virus. They were bled before and after the 16-hour exposure to mosquitoes and both bloods were titered to provide the virus titer range.

† Number of individual mosquitoes infected/mosquitoes tested.

‡ NT = not tested.

§ Number of virus transmissions/number of individual mosquitoes engorged on susceptible chicks.

‖ No. of virus transmissions/No. of *infected* mosquitoes engorged on susceptible chicks.

*mouse-infectious virus in the mosquito and the ability to transmit virus.* On the day after viremic blood was ingested by colonized *C. tritaeniorhynchus*, JE virus was usually detectable in triturated mosquitoes (Table 3). This virus was interpreted as being residual virus from the ingested animal blood. During the next 3 days, virus was often not demonstrable when the mosquitoes were ground and inoculated into mice (Lots III and V, Table 3). This eclipse period of virus will be discussed in more detail elsewhere. (Gresser, *et al.*, in preparation). Usually virus again became detectable during the period from 5 to 9 days after ingestion of viremic blood, but transimssions frequently did not occur until the fifteenth day. This temporal discrepancy between the development of infectious JE virus in *C. tritaeniorhynchus* and the ability of the mosquito to transmit virus to chicks is illustrated in Table 5.

These data demonstrated that a lag existed between the time when virus could be recovered from individual mosquitoes and the time the

TABLE 4

*Comparison of the titer of JE virus in individual* C. tritaeniorhynchus *transmitting virus to susceptible chicks and those failing to transmit*

| Extrinsic incubation (*days*) | No. of mosquitoes transmitting virus / No. infected mosquitoes engorging on susceptible chicks | Titer* of JE virus in infected mosquitoes | | | |
|---|---|---|---|---|---|
| | | Mosquitoes not transmitting | | Mosquitoes transmitting | |
| | | Geom. mean | Range | Geom. mean | Range |
| 10–14 | 0/6 | 4.5 | 3.9–5.1 | — | — |
| 15–19 | 8/22 | 4.9 | 3.8–6.0 | 5.1 | 4.6–5.6 |
| 20–24 | 4/10 | 5.3 | 4.3–6.3 | 5.2 | 4.8–6.0 |
| 25–29 | 13/15 | 4.9 | 4.8–5.0 | 5.1 | 3.9–5.8 |
| 30–34 | 1/1 | — | — | 5.1 | — |

* Titer is expressed as the negative logarithm of weanling-mouse-$LD_{50}$.

TABLE 5

*Temporal relationship between the development of infectious JE virus in* Culex tritaeniorhynchus *and ability to transmit virus**

| Days after exposure to virus | No. of individual mosquitoes infected | No. of transmissions resulting | Per cent of transmission |
|---|---|---|---|
| 10–14 | 8 | 0 | 0 |
| 15–19 | 23 | 10 | 43 |
| 20–24 | 14 | 8 | 57 |
| 25–29 | 26 | 24 | 92 |

* Mosquitoes tested individually.

TABLE 6

*Temporal relationships between development of infectious JE virus in groups of* C. tritaeniorhynchus *and ability to transmit virus*

| Days after exposure to virus | Groups tested | Total mosquitoes | Groups containing one or more infected mosquitoes engorging on a susceptible chick | Transmissions | Transmissions/infected mosquitoes |
|---|---|---|---|---|---|
| 5–9 | 9 | 102 | 7 | 1* | 3/23 |
| 10–14 | 19 | 202 | 16 | 2 | |
| 15–19 | 17 | 114 | 13 | 5 | 17/25 |
| 20–24 | 14 | 55 | 9 | 8 | |
| 25–29 | 9 | 21 | 3 | 4† | |

* Transmission occurred on the ninth day. The mean titer of virus in the blood of the chick host was log 1.9 (16-hour range 2.2–1.6) at time of mosquito feeding.

† In one group of four mosquitoes, no mosquito had visibly engorged and, therefore, no mosquitoes were sacrificed to test for virus. The exposed susceptible chick was, nevertheless, kept and 14 days later, demonstrated HI antibody.

same mosquitoes were able to transmit virus. Further evidence of this discrepancy came from experiments in which small groups of infected mosquitoes, rather than individual mosquitoes, were exposed to susceptible chicks (Table 6). Again, if only groups of mosquitoes from which virus was subsequently recovered were considered as capable of transmitting virus, rather than the total numbers of groups tested, there were only three transmissions in 23 groups before the fifteenth day but 17 transmissions in 25 groups after the fifteenth day (Table 6).

## DISCUSSION

These studies demonstrated that mosquitoes from the twelfth to seventeenth generations of a laboratory colony of *C. tritaeniorhynchus* transmit JE virus to natural hosts such as birds and pigs. When considered with the numerous recoveries of JE virus from *C. tritaeniorhynchus* captured in nature in Japan, this species of mosquito was established as an important, and probably the major, natural vector of JE virus in Japan. The ability of *C. tritaeniorhynchus* in the laboratory to transmit JE virus after ingestion of small quantities of virus in blood of chicks and pigs, suggested that other animals in nature need not circulate virus in high concentrations to infect *C. tritaeniorhynchus*.

During the course of the experiments, two behavioral characteristics of colonized *C. tritaeniorhynchus* were noted. 1) The initial feeding of week-old mosquitoes on animals was often unpredictable while those kept in lamp chimneys for an additional several days to several weeks engorged with less variation. 2) Approximately 50 per cent of the mosquitoes fed within 2 hours of exposure to chicks and only a slight increase in

this percentage occurred in the following 10 hours.

Throughout these experiments only mosquitoes judged by macroscopic examination to be engorged were usually kept for transmission studies. However, virus was recovered from several lots of supposedly "nonengorged mosquitoes" and, on six occasions, transmission of virus to susceptible chicks occurred and none of the mosquitoes appeared to have been engorged. This is in accordance with the experimental observations of Barnett (1956) with Western equine encephalitis virus infection of *C. tarsalis* Coq., and suggests that a mosquito can transmit virus by probing or by ingestion of too little blood to be seen macroscopically. Thus, although ingestion of sufficient blood to be visible macroscopically is a convenient method of judging whether a mosquito has bitten an animal, it is not a completely accurate measure of whether virus was inoculated by the mosquito into the host.

There was no evidence to suggest that mosquitoes from the colony were infected by JE virus prior to exposure to viremic chicks. Since those used for experiments were beyond the twelfth generation from the original mosquitoes caught in nature, their infection by JE virus was highly unlikely unless the virus was transferred transovarially. To be certain that these mosquitoes were not inherently infected, 22 groups of 730 mosquitoes were tested in suckling mice. No virus was recovered.

Why a discrepancy existed between the time virus could be recovered from mosquitoes and the time they were able to transmit virus, and why certain infected mosquitoes apparently failed to transmit virus are two questions that cannot be answered with certainty. Instances did occur when an infected mosquito engorged on a *susceptible* chick whose serum contained no HI antibody upon testing 21 days later. Such failures to transmit virus were not related to differences in total virus present in the mosquito (Table 4). It is possible, however, that had individual organs, such as the salivary glands, been tested, differences in the anatomical distribution of virus might have been found between infected mosquitoes that transmitted and those that did not transmit. Other possibilities, at the moment, include: a) certain *C. tritaeniorhynchus* become infected by JE virus and never transmit virus; b) some chicks supposedly free of maternal antibody by neutralization tests still retain in-vivo immunity; c) a mosquito maintained on slices of apple in the intervals between exposure to susceptible chicks might inoculate the apple with virus and not replenish the supply of virus in its salivary glands soon enough to infect a chick; and d) these observations are either peculiarities of colonized and not "wild" *C. tritaeniorhynchus* or results of the laboratory techniques employed. Further studies are needed to clarify these points.

When the results of transmission attempts from viremic pigs were compared with those from viremic chicks, a discrepancy was noted between the overall percentages of mosquitoes transmitting virus from pigs and from chicks. Virus was transmitted by 92 per cent and 83 per cent of pig-infected mosquitoes, compared with 45 per cent, 48 per cent, and 57 per cent transmissions from chick-infected mosquitoes (Table 3). These results were thought to be related to the few attempts made to demonstrate transmission of virus by pig-infected mosquitoes during the first 19 days after exposure to viremic blood. Had such attempts been made in greater number, more failures to transmit virus with pig-infected mosquitoes would probably have been encountered and the overall transmission rate of mosquitoes infected from pigs would have been lower and nearer the values observed with chicks. This discrepancy was probably due to experimental design and not to a real difference in the percentage of mosquitoes transmitting virus after infection from pigs as compared with chicks.

The ease with which *C. tritaeniorhynchus* transmitted JE virus to chicks in these experiments cannot be taken to indicate that these mosquitoes commonly infect chickens in nature. In fact, surveys for JE antibodies during 1946 showed that chickens in Japan, Okinawa and Korea infrequently had detectable antibodies (Bawell *et al.*, 1950; Deuel *et al.*, 1950). Moreover, during 1955 and 1956, hens in the Tokyo area could not have been infected by JE virus in high incidence since only 10 per cent of newly hatched chicks had maternal neutralizing antibody (Gresser, unpublished data). The explanation for the discrepancy between the low natural infection rate of chickens and the ease with which chicks became infected by virus from *C. tritaeniorhynchus* in the laboratory, of course lies in the zoophilism of *C. tritaeniorhynchus* under natural conditions. Sasa and Sabin (1950) showed

that chickens attracted relatively few *C. tritaenorhynchus* when compared with persons, goats, and rabbits, and Buescher (personal communication) found that *C. tritaeniorhynchus* entered chicken-baited traps much less frequently than traps containing BCNH, a bird that serves frequently as a natural host of JE virus. Data such as these emphasize again that the behavior of mosquitoes in nature must always be considered while intrepreting the results of laboratory experiments.


## SUMMARY

*C. tritaeniorhynchus*, the principal mosquito infected in nature by Japanese encephalitis virus in Japan, was shown, after colonization and propagation for 12 to 17 generations in the laboratory, to transmit JE virus readily from chick to chick and from pig to pig, to Black-crowned Night Heron, to egrets and to baby chicks. Mosquito infection and transmissions followed ingestion of blood containing as little as three mouse-$LD_{50}$ of JE virus/0.04 ml blood (0.15 $LD_{50}$/mosquito). The concentration of virus in blood of the host chick or pig governed the numbers of mosquitoes infected and, consequently, the overall transmission rate, although some infected mosquitoes failed to transmit virus. Virus was recovered from mosquitoes within several days after feeding on a viremic animal, but transmission usually did not occur until more than 2 weeks (range of 9 to 34 days) after ingestion of viremic blood.



## ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Messrs D. Baver, M. Nonga, K. Hata and the cooperation of the Dept. of Entomology, 406th Medical General Laboratory, for supplying egg rafts from the colony of *Culex tritaeniorhynchus* for these experiments.


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# DAILY PERIODICITY OF *SCHISTOSOMA MANSONI* CERCARIAE IN PUERTO RICAN WATERS[1]

WILLIAM B. ROWAN[2]

Recently, the writer described the construction and operation of an apparatus which permits the rapid quantitative recovery of *Schistosoma mansoni* cercariae from relatively large samples of flowing and impounded water (Rowan, 1957). The device has been used in the study of cercarial periodicity in three Puerto Rican streams and a pond. This paper summarizes about 100 cercariae determinations, the results of which may be of use to persons concerned with health education and other phases of schistosomiasis control.

## METHODS

The cercaria recovery device used in these experiments consists of a pressure filtration chamber connected by rubber hose to a manually-operated water pump (Figs. 1 and 2). The water to be examined is drawn from the infective source, through the intake hose, to the pump. Passing through the pump it enters the filtration chamber where it is forced under pressure through a selected grade of filter paper (S and S 404) which traps cercariae, silt, zooplankton, and other suspended matter. The filtered water is collected in a large metal can which has graduated markings. When a sample of desired volume (5 to 30 gallons) has been pumped through the apparatus and only air remains in the pressure chamber with the paper, the damp paper is removed from the chamber, transferred to a shallow pan containing 5 ml of 0.5 per cent ninhydrin reagent and heated until dry over an improvised alcohol stove. The sample volume, date, location, time of day, and other pertinent information are recorded on the dry paper. Each complete test requires from 10 to 20 minutes and the paper disc can be studied immediately or stored for as long as 6 months before it is examined.

Accurate total counts of the cercariae on the paper are made in the laboratory by drawing parallel pencil lines across the paper, placing it between glass plates, saturating it with xylol containing excess sodium bicarbonate, and examining it with a dissecting microscope using reflected light and magnifications of 9× and 36×. The xylol makes the paper translucent and each *S. mansoni* cercaria appears dark blue with a reddish acetabular region against the brownish, unstained silt background. A trained technician requires from 5 to 15 minutes to examine a paper completely.

The volume of sample which can pass through the paper depends on the turbidity of the water under study. When the water contains less than two parts per million of suspended material, 20 to 30 gallons can be filtered. At a turbidity of 15 ppm, the maximum sample volume is only 5 gallons. If the turbidity exceeds 15 ppm, the water sample must be collected in a large can and the suspended silt and debris flocculated with commercial filter alum before it can pass through the apparatus.

Details of the technique, using the apparatus for direct pumping from the source (direct samples) as well as in conjunction with the alum-flocculation procedure (alum samples) were presented in the original description (Rowan, 1957). This description includes a discussion of the experiments done to establish the degree of efficiency and consistency with which the apparatus traps cercariae in samples varying in both volume and turbidity. These experiments showed that when direct samples are taken of water containing 0 to 16 ppm suspended materials, one can expect an average of 45 ± 8 per cent of the cercariae in the sample to appear on the paper sufficiently well oriented to be easily recognized as *S. mansoni*. Actually, an average of 64 per cent of the cercariae in the sample are recovered, but some of these are broken in half and some are only partially visible deep down in the fibers of the paper. Only those easily recognized as *S. mansoni* are counted (Fig. 3). Consequently, to arrive at a fairly accurate determination of the true number of

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FIG. 1

FIG. 2

FIG. 3

cercariae per unit volume of water examined, the total number of easily-recognized cercariae on the paper is multiplied by 100/45 and divided by the volume. Sample volumes, in this study varied from 8.5 to 30 gallons and cercarial densities are reported as cercariae per gallon.

When high turbidities are encountered and the alum-flocculation procedure is included in the sampling technique, the total expected recovery is 36 per cent, and an average of 23 $\pm$ 6 per cent of the cercariae in the sample are sufficiently well oriented on the paper to be easily recognized as *S. mansoni*. The cercarial density for alum-flocculated water is determined by multiplying the total number of cercariae on the paper by 100/23 and dividing by the volume. When using alum flocculation, a standard sample of 15 gallons is taken. In this report, the technique, whether direct or alum sampling, is specified for each series of data presented.

Samples used to determine cercarial density were taken hourly from two rivers (Rio Caguitas, Rio Cañas) and one stream (Quebrada Placita); and every 1.5 hours from a pond (at Mayaguez). Preliminary trials at the Rio Cañas and Quebrada Placita had shown that the cercariae were

about equally distributed at various depths in shallow streams. At Rio Cañas ten trials also showed that the most constant recovery rates in flowing water were obtained from the main channel rather than at the edges, in eddies, or other locations. Therefore, all of the stream samples in the present study were taken from the upper 6 inches in the main channel.

Various precautions were used at Mayaguez to secure representative pond-water samples. The possibility that cercariae might stratify vertically was considered. The writer studied the vertical movements of cercariae in 1-L graduated cylinders in the laboratory during the day and night and noted that while the cercariae maintain a fairly uniform distribution during the daytime, they show some tendency to congregate near the bottom at night (from 11 p.m. to 7 a.m.). They resume a uniform pattern of distribution soon after daylight returns, or uniform distribution can be induced at night by using artificial light. The Mayaguez samples were collected in 1 foot of water, 2 to 5 feet from shore.

On October 16, the first day of study at Mayaguez, samples were dipped from the pond with a 3-gallon pail and transferred to a large can for flocculation, taking care to dip deeply and at several points along a 20-foot sector of the shore. On the second day of study, November 19, an auxiliary pump was used to draw water from the pond into the flocculation can. While pumping, the end of the intake hose was placed at different depths and at various positions along the shore. Undoubtedly, this auxiliary pump destroyed some cercariae but, since it was used consistently in collecting this series of data, the hourly fluctuations in cercarial density for the series are comparable. When the alum technique was used at night, artificial light was used over the flocculation chamber to reduce the tendency of the cercariae to congregate near the bottom.

Following each day of testing with the cercaria-recovery apparatus, the abundance of the host snail, *Australorbis glabratus*, in the area was estimated with the aid of a 0.5-square-foot screen dipper. The snails were examined in the laboratory to determine the per cent infected with *S. mansoni*.

The volumes of flow of streams at or near the sampling point were calculated using the Embody formula as outlined by Welch (1948).

## DESCRIPTION OF THE STUDY AREAS

*Quebrada Placita.* Quebrada Placita is a small stream of moderate gradient (average for 1 mile, 2.5 per cent grade) which flows through the community of Placita and joins the Rio Gurabo approximately 1 mile north of Juncos. Water samples varying in volume from 8.5 to 10 gallons were collected and examined hourly on April 24, 1957 from 9 a.m. to 4 p.m.; on April 25 from 6 a.m. to 10 a.m.; on April 29 from 7 a.m. to 7 p.m.; and on May 8 from 7 a.m. to 11 a.m. at a point 100 feet upstream from the Gurabo River junction. At this point on April 24 the stream had an average depth of 3.5 inches, an average width of 4.5 feet, a surface velocity of 1.3 feet per second, and an approximate volume of flow of 2.1 cubic feet per second. During April, *A. glabratus* snails were present in large numbers (over 20 per square yard) in the lower 300 yards of the stream. Thirteen per cent of the snails collected on April 29 were found to be infected.

*Rio Cañas.* The Rio Cañas is a small river of 1.5 per cent gradient north of Caguas. Water volumes varying from 10 to 20 gallons were collected and examined hourly on May 1 from 7 a.m. to 1 p.m. at a point 400 feet southwest of a bridge on Highway 1 (San Juan to Caguas). At this point on May 1st the stream had an average depth of 5 inches, an average width of 8.5 feet, a surface velocity of 1.4 feet per second, and a volume of flow of about 5.9 cubic feet per second. *A. glabratus* snails were present in moderate numbers (5 to 10 per square yard) for several hundred yards upstream from the sampling point, but infected snails were collected from only one position, approximately 500 feet upstream. One per cent of the snails at this position harbored *S. mansoni*.

*Rio Caguitas.* The sampling station on the Rio Caguitas, at Caguas, was located 300 feet east of a bridge on Highway 1. At this point the river is of low gradient (0.8 per cent) and varies from 20 to 30 feet in width and from 1 to 3 feet in depth, with a surface velocity of approximately 0.6 feet per second. Measurements taken upstream, beneath the highway bridge, on May 7 indicated the volume of flow to be about 22.5 cubic feet per second. *A. glabratus* snails were present but not abundant (one per square yard in marginal vegetation), and no infected snails were found for a distance of 500

TABLE 1

*Daily periodicity of* Schistosoma mansoni *cercariae in Puerto Rican waters*

| Hour | Cercariae per gallon | | | | | | | | | Hour |
|---|---|---|---|---|---|---|---|---|---|---|
| | Quebrada Placita | | | | Rio Caguitas | Rio Cañas | Mayaguez Pond | | | |
| | April 24* | April 25* | April 29* | May 8† | May 7* | May 1* | Margin Oct. 16† | Outlet Oct. 16† | Margin Nov. 20 and 19† | |
| *A.M.* | | | | | | | | | | *A.M.* |
| 1 | — | — | — | — | — | — | 0 | 0 | 4.6 | 1:00 |
| 2 | — | — | — | — | — | — | 0.6 | 0 | 3.5 | 2:30 |
| 3 | — | — | — | — | — | — | | | | |
| 4 | — | — | — | — | — | — | 0 | 0 | 4.5 | 4:00 |
| 5 | — | — | — | — | — | — | 0 | 0 | 2.9 | 5:30 |
| 6 | — | 0 | — | — | — | — | | | | |
| 7 | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.3 | 7:00 |
| 8 | — | 0 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0.3 | 8:30 |
| 9 | 1.6 | 0.2 | 1.3 | 0.4 | 0 | 0 | | | | |
| 10 | 4.7 | 9.1 | 11.1 | 15.4 | 0.33 | 0.20 | 1.6 | 0.3 | 0.9 | 10:00 |
| 11 | 135.0 | — | 67.8 | 20.0 | 0.78 | 0.40 | 27.2 | 19.4 | 32.2 | 11:30 |
| Noon | 59.0 | — | 56.7 | — | 1.45 | 0.90 | | | | |
| *P.M.* | | | | | | | | | | *P.M.* |
| 1 | 28.2 | — | 16.4 | — | 0.78 | 0.20 | 40.0 | 46.3 | 63.0 and 46.2 | 1:00 |
| 2 | 16.9 | — | 12.0 | — | 0.15 | — | 25.5‡ | 26.0‡ | 85.0 | 2:30 |
| 3 | 27.2 | — | 1.6 | — | 0.33 | — | | | | |
| 4 | 3.6 | — | 0 | — | 0.22 | — | 70.7 | 46.5 | 103.0 | 4:00 |
| 5 | — | — | 0 | — | 0 | — | 44.0 | 7.8 | 74.0 | 5:30 |
| 6 | — | — | 0.4 | — | 0 | — | | | | |
| 7 | — | — | 0.4 | — | 0 | — | 39.7 | 15.6 | 20.3 | 7:00 |
| 8 | — | — | — | — | — | — | 29.5 | 6.4 | 15.7 | 8:30 |
| 9 | — | — | — | — | — | — | | | | |
| 10 | — | — | — | — | — | — | 14.7 | 7.3 | 10.5 | 10:00 |
| 11 | — | — | — | — | — | — | 18.3 | 2.6 | 4.1 | 11:30 |
| 12 | — | — | — | — | — | — | | | | |

* Direct sampling: intake hose to the pump was placed directly into main flow of stream.

† Alum sampling: filter-alum was used to flocculate suspended silt before sample was passed through paper.

‡ Sampling failure: filter-alum failed to produce suitable floc; only part of sample filtered; figures for cercarial density are undoubtedly lower than actual.

feet upstream. The water volumes examined hourly on May 7 from 7 a.m. to 7 p.m. varied from 20 to 30 gallons.

*Mayaguez Pond.* The pond studied is one of a series of ponds on the north edge of the town of Mayaguez. These ponds are connected by concrete channels, but during periods between heavy rains very little water flows through them. They vary in size, the study pond being approximately 100 feet long, 20 to 40 feet wide, and 1 to 3 feet deep. *A. glabratus* snails were abundant (10 to 20 per square yard) at most points in this pond at the time these tests were made, and nearly 50 per cent of the snails collected were found to be infected with *S. mansoni*. Water samples (15-gallon, alum flocculation) were collected at 1.5-hour intervals at two positions in the pond on October 16 from 1 a.m. to 11:30 p.m., and at one position from November 19 at 1 p.m. to November 20 at 1 p.m.

## RESULTS OF THE CERCARIAL DENSITY DETERMINATIONS

The relationship between cercarial density in each of the study areas and the time of day is presented in Table 1 and Figures 4 and 5. A marked fluctuation in density is evident at all four locations. In the stream and river samples

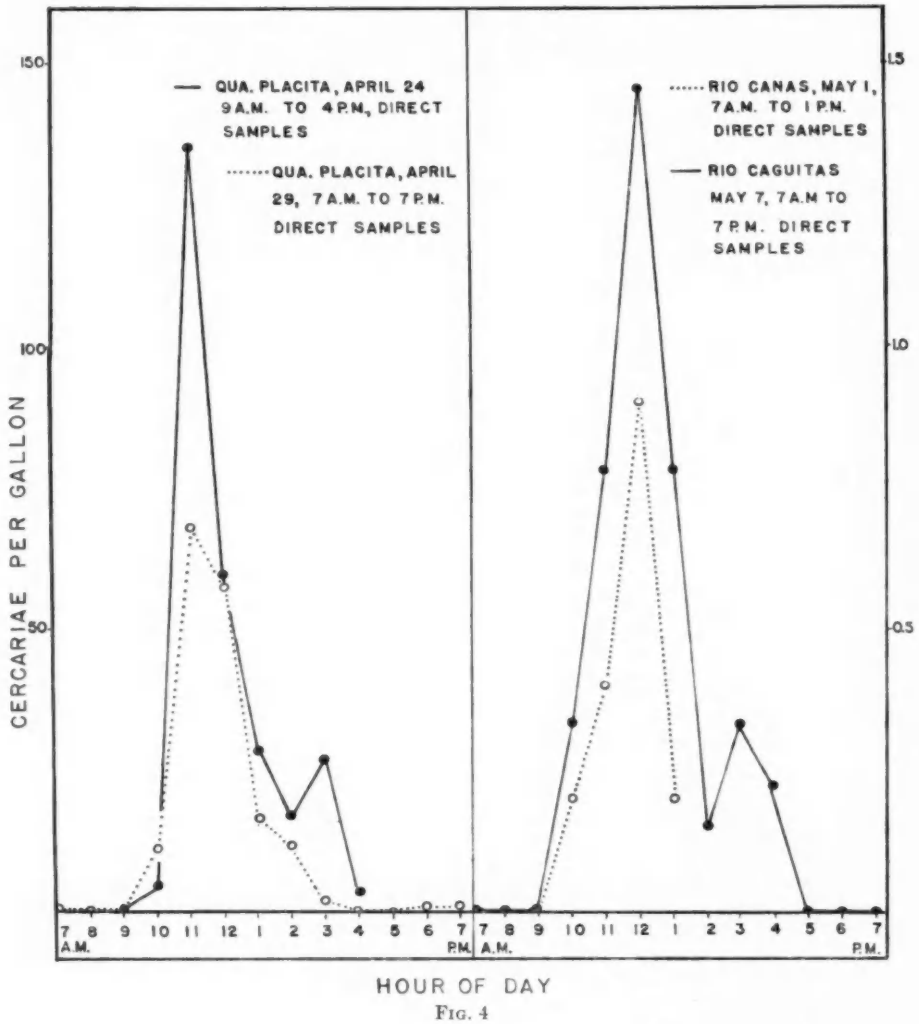
QUA. PLACITA, APRIL 24 9A.M. TO 4P.M., DIRECT SAMPLES
QUA. PLACITA, APRIL 29, 7A.M. TO 7P.M. DIRECT SAMPLES
RIO CANAS, MAY 1, 7A.M. TO 1P.M. DIRECT SAMPLES
RIO CAGUITAS MAY 7, 7A.M TO 7P.M. DIRECT SAMPLES
CERCARIAE PER GALLON
150
100
50
1.5
1.0
0.5
7 8 9 10 11 12 1 2 3 4 5 6 7
A.M.
P.M.
HOUR OF DAY

FIG. 4

the peak of cercarial abundance lies between 11 a.m. and 1 p.m. with few cercariae present in the water before 9 a.m. or after 4 p.m. On November 19 and 20 the cercarial density in the Mayaguez pond was found to reach a peak at 4 p.m., to decline rapidly until 8:30 p.m. and then to continue to decline gradually throughout the night to a low at 8:30 a.m. the following morning.

The experiment at Mayaguez on October 16 was conducted following unusual weather conditions. A heavy rain storm on the afternoon of October 15 caused the pond to flood for several hours. By midnight the flood had subsided and sampling was begun at 1 a.m., October 16. Although 20 gallons of water were filtered every 1.5 hours from 1 a.m. throughout the day, practically no cercariae were recovered from the water until the usual 10 a.m. rise in density.

At the study areas where repeated tests were made, the maximum density of cercariae per gallon varied from day to day. The cercarial

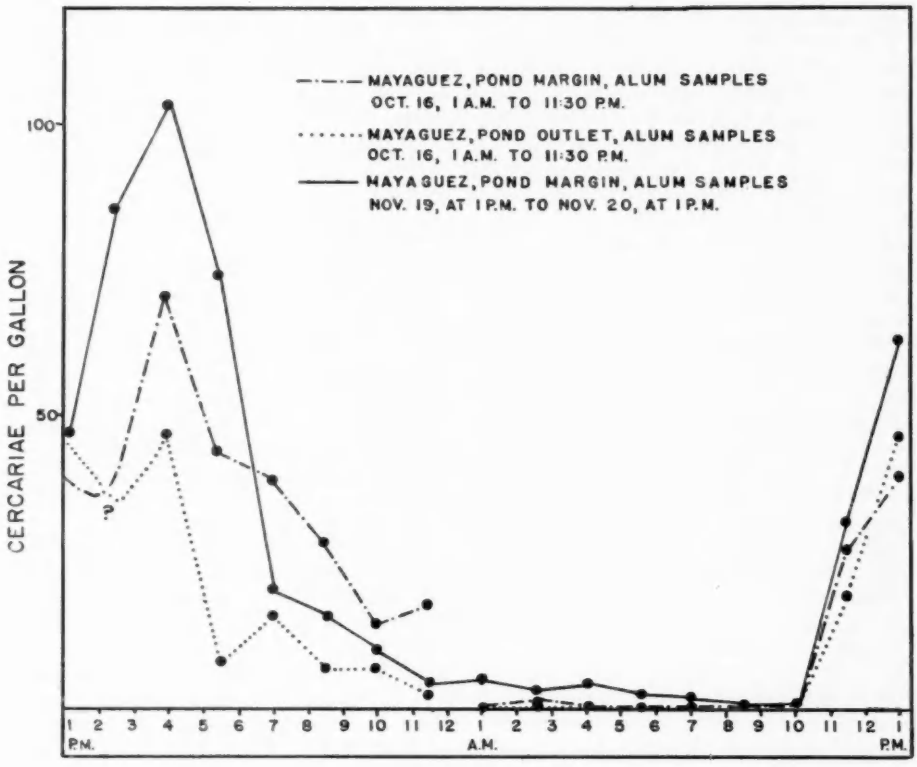
MAYAGUEZ, POND MARGIN, ALUM SAMPLES
OCT. 16, 1 A.M. TO 11:30 P.M.
MAYAGUEZ, POND OUTLET, ALUM SAMPLES
OCT. 16, 1 A.M. TO 11:30 P.M.
MAYAGUEZ, POND MARGIN, ALUM SAMPLES
NOV. 19, AT 1 P.M. TO NOV. 20, AT 1 P.M.
CERCARIAE PER GALLON
100
50
?
1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1
P.M.
A.M.
P.M.
HOUR OF DAY

FIG. 5

density at 11 a.m. at Placita on April 24 was almost twice that of the sample taken at the same time April 29. A record density of 240 cercariae per gallon was obtained at Mayaguez on October 15 at 2 p.m.

In these experiments mice or other animals were not routinely exposed to the water at the sampling sites to provide supporting data for the density determinations. Dual testing of this type has been done in other experiments designed for other purposes. However, two groups of five mice were exposed to the pond water at Mayaguez on November 20 from 3 a.m. to 4:30 a.m., and from 7 a.m. to 8:30 a.m., respectively. This was done by carefully suspending cages over the pond in such a way that the mice were forced to remain partly immersed in the water at all times. One mouse from each group died of infection resulting from bite wounds 2 weeks after exposure. The surviving mice were sacrificed and examined 7 weeks after exposure. Although the cercarial density during the exposure period varied from 3.5 to 0.3 cercariae per gallon (Table 1), no worms were recovered from any of the mice. Their livers were normal in appearance, and washings from the ground intestinal and liver tissue of the two groups contained no schistosome eggs.

## DISCUSSION

It is not known why the release of *S. mansoni* cercariae from the snails in the field begins abruptly in midmorning. Light and temperature were considered as possible factors and readings of these factors were taken at most of the study areas. On every day studied except one (May 8,

Placita) the weather was clear and warm during the test period, but on that day it rained steadily from 6 a.m. until noon. The sun did not appear and no measurable change was noted in the water temperature during the morning. Nevertheless, the pattern for release of cercariae from 6 a.m. to 11 a.m. of that day was similar to the pattern observed at Placita on April 24, 25, and 29 (Table 1).

On the basis of the data obtained at Mayaguez, October 16, where testing began at night following a severe flood, it would appear that no cercariae, or an insignificant number, are released during the night. The release of cercariae from snails in the field apparently occurs primarily from 9 a.m. to 4 p.m. with the most rapid emergence at noon. This conclusion supports the laboratory findings of Faust and Hoffman (1934), Giovannola (1936) and others working in Puerto Rico. Faust and Hoffman reported that the cercariae emerge in the laboratory from 9 a.m. to 2 p.m., particularly when the snails are exposed to sunlight. Giovannola noted that emission of cercariae is highest from 9:30 a.m. to 3:30 p.m.

Faust and Hoffman (1934) and others have shown that *S. mansoni* cercariae normally survive in the laboratory for longer than 24-hour periods. Apparently few, if any, cercariae survive this long under natural conditions. The observed reduction in numbers of cercariae in the streams and pond following the period of emergence from snails is probably caused by a variety of factors. In streams the cercariae are carried away rapidly from their point of release. Mechanical injury due to turbulence and predation perhaps combine to reduce their numbers downstream. The possibility of mechanical injury by turbulence was demonstrated in the laboratory for *S. japonicum* cercariae by Suguira and Ota (1954). Comparable information for *S. mansoni* cercariae is not available at present. The writer has observed predation of *S. mansoni* cercariae by the guppy (*Lebistes* sp.) which was present in abundance in the streams and pond studied. In one experiment in the laboratory, four *Lebistes* in 1 L of water consumed over 7,000 *S. mansoni* cercariae in less than 3 hours. The fish ate continuously and no cercariae remained in the water after 3 hours. The importance of predation as a factor in limiting the numbers of cercariae in ponds and streams is a subject which deserves additional investigation.

The peak of cercarial density in the pond at Mayaguez occurred several hours later than in the streams studied. Probably, the cercariae released in the pond accumulate for several hours, few being washed away by flowing water as in the streams studied. One might expect that their abundance in the pond would increase until the fatality rate due to predation and/or other factors exceeds the rate at which they are shed.

No definite reason can be advanced for the fact that mice, exposed to the Mayaguez pond water in the early morning on November 20, failed to become infected even though cercariae were present in the water. It is possible that the cercarial density was too low at that time to establish a recognizable infection in the animals. However, it is also possible that cercariae, remaining in the water from a previous day's emergence, are too old to penetrate a suitable host and establish infections.

The cercarial periodicity data have practical applications for persons concerned with health education of the public in Puerto Rico with respect to schistosomiasis. The writer has noticed that members of some families consistently draw their water for drinking or do their washing and bathing at hours which coincide with the peak of cercarial abundance, while members of other families quite consistently select other times of day for these activities. Slight changes in the time of water usage on the part of some families might materially reduce their exposure to cercariae. The safest period for use of water would appear to be from 5 a.m. to 9 a.m.

Judging from the work at the Rio Caguitas, where no infected snails were found after considerable searching, but where cercariae were recovered from the water, it would seem that the cercaria-detection apparatus and technique used in these experiments is a more rapid, sensitive, and accurate means of survey in schistosomiasis studies and control projects than snail collection and isolation procedures. The method might be used to determine the relative importance of various foci within a schistosomiasis control zone, thereby permitting more economical and effective use of disease control funds.

## SUMMARY

The density of *Schistosoma mansoni* cercariae in Puerto Rican waters was found to undergo a

marked daily fluctuation. In the rivers and stream studied, the peak of cercarial abundance was found to occur between 11 a.m. and 1 p.m. Few cercariae were present in the water before 9 a.m. or after 4 p.m. In a heavily infested pond, cercariae appeared at 10 a.m. and their numbers accumulated to a peak at 4 p.m. After 4 p.m., the density declined rapidly at first and then more slowly to a low at 8:30 a.m. the following morning. On the basis of these experiments, the hazard of exposure to cercariae in Puerto Rican waters is considered to be from 20 to 100-fold less between the hours of 5 a.m. and 9 a.m. than at most other times of the day.

The recovery apparatus and technique used in these experiments are suggested as an accurate timesaving device for stream survey work, and as a useful aid to the development of more economical means of schistosomiasis control.

ACKNOWLEDGEMENTS

The writer is grateful to the Carl Schleicher and Schuell Company, Keene, New Hampshire, and the Eaton Dikeman Company, Mt. Holly Springs, Pennsylvania, for providing experimental quantities of various grades of filter paper. He wishes to thank Mr. Anibal Carrion and Mr. Tomas Ocasio of the Puerto Rico Department of Health, for their assistance in collection of the water samples.

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# A NEW CONCEPT IN MICROSCOPE DESIGN FOR TROPICAL MEDICINE

JOHN McARTHUR[1]

A microscope specially designed for tropical medicine must have certain particular qualities. It must be not only optically as perfect as possible but exceptionally robust to withstand the rough treatment of travel, light, and easily portable. It must be resistant to heat, humidity and dust, and capable of every operation within the range of any high-power microscope.

The instrument described in this article represents a completely new concept in microscope design and, together with a complete range of accessories, is considered to be practical and capable of undertaking any work within the range of the largest conventional instrument, and of undertaking some forms of work beyond the ability of any other microscope.

This microscope is based upon a prismatic system and a completely new design of stage. Fully equipped with standard low and high power and oil-immersion objectives, eyepiece and condenser, and with attachments for all accessories, it measures only 4 x 2½ x 2 inches, and weighs only 18 ounces. It is thus about the size and weight of a modern miniature camera and, being a massive block of metal, it is extremely rigid and readily portable.

The instrument consists essentially of a sturdy rectangular casing, as shown in Figure 1. Light enters this casing from above, either reflected from an external source of light by a stainless steel mirror which also forms a dust cover, or directly from a new design of built-in electric illuminator. The light passes vertically downwards through a condenser to the ordinary 3″ x 1″ slide, which lies face downwards on the stage with the specimen in a recess. From the specimen the light passes downwards into the upwardly-directed objective. The beam from the objective forming the primary image continues downwards into a prism, is then reflected horizontally along the base of the instrument, and finally upwards by a second prism into the downwardly-directed eyepiece.

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The microscope has standard objectives except that their metal bases have been removed to save space, a standard eyepiece, and the ordinary tubelength, so that the image is exactly the same as that in the conventional microscope except that it has the advantage of being erect instead of inverted. Three objectives can be accommodated on their carrier, with a range from the lowest to the highest powers including oil-immersion lenses, and each slides easily and simply into place.

The condenser, the stage and the objective are thus all inverted in comparison with the conventional microscope, and this, far from having disadvantages, is found to have a number of real advantages; the chief of which being that, since the slide is also inverted, and with the specimen therefore always in the plane of the stage whatever the slide thickness, focusing is automatic. All normal specimens are in focus immediately they are placed on the stage, even with the oil-immersion lens. It is possible to run through a whole series of blood films, for example, without the necessity for more than half a turn of the fine adjustment to sharpen up the details. It is impossible to push the objective through any normal specimen, and it is unnecessary to rack the objective away from the specimen with a change of slide. Thus, for laboratory work, examination is faster and safer than with the conventional microscope.

Although designed for critical research and general-purpose work in the laboratory, this microscope has the unique advantage that it can also be used in the hand. Thus, examinations can be made, with the highest powers, and with its own built-in electric lighting for bright-field and even dark-field illumination, where there is no bench, or where there is vibration, such as while travelling by air (Figure 2).

A small degree of fine adjustment is provided, and this is achieved not by the usual sliding dovetail, which is liable to wear, and to lubrication troubles, but by a simple arrangement of parallel springs, which provides a smooth motion,

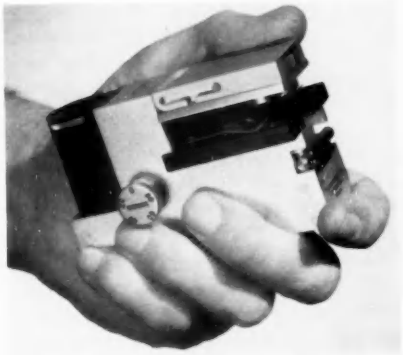

FIG. 1. *The complete microscope.* Equipped with three standard objectives, eyepiece and condenser, the instrument can be carried in the pocket, and used in the hand for the highest power work.

FIG. 2. *The microscope in flight.* In a C.A.T. plane over Formosa (the curtains drawn for security reasons), presumably the first identification in the vibration of an aircraft, of malaria parasites in a blood film, is made by Dr. John W. Field, lately Director of the Institute for Medical Research, Kuala Lumpur. Behind Dr. Field is Professor Morishita, University of Osaka, and Dr. E. J. Pampana, Chief of Malaria Section, World Health Organization. To Dr. Field's left is the designer.

Photo by Dr. Raymond Laird, Chief Malariologist, US FOA, Indonesia.

with no sliding friction, no lubrication, and no possibility of wear or backlash.

Not only are all the objectives in focus, but different series of objectives may be rapidly interchanged without disturbing the focus. Thus, it is possible to mount three dry objectives, say 10×, 20× and 40×, on one interchangeable plate, and three immersion objectives, 50×, 95× fluorite, and 95× stopped-down for darkground work, on another, all precisely centred and parfocal. Again, since the objectives are always in focus, all other optics, such as condensers for both bright and dark fields, light source, and a variety of accessories, are instantly interchangeable and always remain clamped precisely and rigidly in adjustment.

For laboratory work this instrument can be mounted on a stand, equipped with interchangeable binocular tube, demonstration eyepiece, and comparison eyepiece. Corrected oil-immersion condenser, phase contrast, and incident illumination for solid objects are now being arranged.

One of the most useful accessories for this microscope is an efficient, built-in, electric illuminator, little larger than a matchbox, operating from self-contained long-life or external batteries, or by a tiny transformer direct from the mains, and sufficiently intense for high-power and darkground illumination and even for projection.

A number of unusual operations can be carried out with this microscope. With a long-focus objective and supplementary stage it may be used, aided by its erect image, for the dissection of mosquitoes and other insects, following which the dissected material may be examined under a high-power lens. Again, material may be made accessible for staining or microdissection, even with the oil-immersion lens.

The hanging-drop preparation used in bacteriology (which is troublesome to make, difficult to focus, and provides a surface for examination which is curved, vibrates readily, and is seen through a depth of fluid which may be sufficient to disturb the optical corrections of the objective) is replaced by the lying-drop preparation which is simple to make, is at once in focus, and provides a flat surface for examination which does not vibrate and which is seen, as it should be, only through the cover-glass thickness; while sediment can be examined, at any magnification, and in a large volume of fluid.

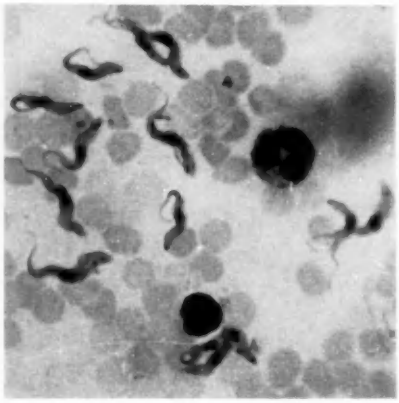

FIG. 3. *Photomicrograph ×2,000 taken by night on a moving bus in Piccadilly, London.* Exposure $\frac{1}{100}$ of a second, using a built-in illuminator and internal battery, with ordinary, achromatic, oil-immersion objective on standard 'McArthur' microscope, and with standard 'Wrayflex' 35-mm camera, bloodfilm with trypanosomes.

Darkground illumination, normally a troublesome operation, can be carried out with ease, the prefocused and precentred condenser simply snapping into permanent adjustment. Lighting by the built-in electric illuminator renders spirochetes as clearly visible as by the normal laboratory equipment, but with the microscope held in the hand, and with no adjustments whatever.

Photomicrography can be carried out with this microscope, using a standard 35-mm camera which is also available for ordinary photography. By a compact and rigid attachment, which at the same time forms a convenient bench stand for the instrument, the image as seen focused in the eyepiece can be photographed, (Figure 3) without interrupting the view, in monochrome or color, singly or in rapid succession, simply by the pressure of a button; if necessary, under the most difficult circumstances it is possible to imagine. In the same way it is possible to attach the microscope by the standard thread to a 16-mm cine camera, in such a way that while a film may be taken of a moving object as seen in the eyepiece, the camera does not interfere with the use of the microscope, nor the microscope with the use of the camera for normal filming.

A completely new form of 'gliding' stage is in prototype, with advantages for tropical and laboratory work; and three forms of binocular are designed: straight, to allow the microscope to be used directly on a bench; inclined, for use with camera or other forms of bench stand; and a special form, developed for tropical work, which is 'tailored' to the individual interocular distance, requiring no adjustment, permanently cemented and sealed against the atmosphere, and permitting the microscope to be held in the hand if required.

Blood counts with this instrument are simple, speedy and accurate, requiring no bench, no focusing, no mechanical stage, and no calculations, yet with sharp ruling even when the diaphragm is fully opened. Red and white cell counts can be easily and accurately carried out with this microscope held in the hand under difficult circumstances.

The prisms directing the beam of light are firmly and permanently cemented in a light tube which is completely closed and dust proof. This can be removed and replaced by other light tubes for special purposes. Special units already available include an erecting tube for dissecting, a beam-splitter tube incorporating a semi-silvered cube for photomicrography and for incident illumination. If the light tube is removed entirely the image can be projected downwards onto the bench for demonstration, counting, measurement and drawing.

The microscope is rather low on the bench for routine work, but a light, rigid tripod raises it to a convenient level and inclines the instrument for comfort and for drawing with a camera lucida.

The entire microscope, equipped for the highest power work, incorporating its own built-in electric illuminator, can be carried in the pocket, or in a

FIG. 4. *For rural surveys.* The microscope may be used with ease under circumstances where no other microscope can be used at all.

neat leather case on the belt, instantly ready for work while travelling or in the field. It may be sealed against complete submersion and protected against humidity and is designed in corrosion-resisting materials for rigorous climates. It is free from lubrication so as to be unaffected by extremes of temperature, and has been used at temperatures ranging from equatorial heat to antarctic cold at −40F. It can be sent economically by air-mail and, for air travel, it has the advantage that in the pocket or on the belt it is not included in one's baggage allowance.

It may be used effectively under the most difficult circumstances. For example, mosquito larvae may be examined when one is stooping, pestered by insects, over breeding places in the jungle; a blood count may be made in the chaos of a native hut (Figure 4); blood films can be examined while standing surrounded by children in a primitive village. Spirochetes may be searched for while standing in the open on a dark night. Smears and sections may be reported on when sitting in a swaying dug-out canoe, on a wind-swept snow ridge, or while travelling in trains, planes and motor cars, with the same perfection of image that it is possible to obtain in the most highly equipped laboratory.

Work on this microscope was begun 25 years ago (McArthur, 1934), was developed largely during the writer's malaria research work in Borneo before the war, and its advances were worked out in prison camp (McArthur, 1945).

Among other distinctions, it was instrumental in the discovery of *Anopheles leucosphyrus* as the vector of malaria in Borneo, which was incriminated by dissection just before World War II (McArthur 1947).

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# THE PREVALENCE OF *SCHISTOSOMA MANSONI* IN CERTAIN LOCALITIES OF PUERTO RICO—A THREE YEAR STUDY[1]

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During the meetings of the W.H.O. Expert Committee on Bilharziasis in San Juan, in November 1952, the need was felt for a careful study of the status of the disease in certain areas of the island. A 3-year program was organized for that purpose, the work starting on February, 1953, and extending until the same month in 1956. The present report gives the results obtained.

## PROCEDURE

*Localities studied.* Six previously-reported foci were selected for investigation (Fig. 1). The six may be considered representative of three physiographic areas of the island (Harry and Cumbie, 1956). Two of them, Barranquitas and Comerío, are located in the same water shed of La Plata River, 1,500 to 2,000 feet above sea level. A single, small and highly polluted, low-gradient stream constitutes the known source of schistosomiasis in Barranquitas. It runs along the edge of town with numerous other dwellings situated along its course above and below the urban area. In Comerío, the source of infection is the main body of La Plata River, dammed several kilometers down stream. Untreated sewage enters directly into the water.

Two other areas, Jayuya and Utuado, are located in narrow valleys higher up in the Central Mountain range. Both are bordered by rivers of rapid flow, which evidently constitute the sources of infection.

The fifth focus, located on the eastern shore of the island, covers the municipality of Ceiba and a ward (Daguao) of the adjacent town of Naguabo. The entire area is drained by numerous small streams originating in the Luquillo Mountain range. These streams have high gradients in the upper reaches, but become sluggish within a mile or two of the coastline. The lower portions of these streams are used intensively by the inhabitants for various domestic purposes.

The sixth locality, Guayama, lies in the dry, artificially-irrigated, southern coastal plain, where sugar cane is practically the only crop grown. The irrigation system is the main source of water for a good portion of both rural and suburban dwellers and constitutes a classical hyperendemic focus of schistosomiasis for Puerto Rico (Hoffman and Faust, 1934).

*Method of sampling.* The investigation was based entirely on fecal examination. The local offices of the Superintendents of Schools and the Public Health Units of the Department of Health helped in the work. Many centers of population were included from each area, but most of the samples came from urban and suburban places. Usually, collection was done once a week, or at the convenience of the collectors, and the tightly covered, paraffin-coated containers were placed in a refrigerator until the sample could be examined.

*Population covered.* A total of 24,585 fecal samples were examined, but information on the name and age of the contributor was obtained for 23,262. Only the latter were used on the final analysis.

Jayuya was the most inaccessible municipality, and correspondingly fewer samples (average, 874) could be collected there in the yearly samples. Among the others, the average per year varied from 1,044 for Barranquitas to 1,652 for Comerío.

Between 70 and 75 per cent of the individuals were below 15 years of age; the distribution by sex among them being about equal. Among the adults (16 years of age or over) there was a tendency for females to cooperate more willingly. For the 21- to 25-year group the ratio was five females to one male, but the disparity diminished until beyond 41 years of age, when it was only 1.5 to 1.

The great majority of the samples came from the lower economic levels, since only a small proportion of the population within the higher income brackets attends public schools or the dispensaries of the Department of Health. In some places, as in Comerío and Utuado, many samples came from workers in the tobacco factory, mostly female. Sampling in other lo-

[1] Carried out under Contract No. DA-40-007-MD-424 with the Office of the Surgeon General of the U. S. A. Department of Defense.

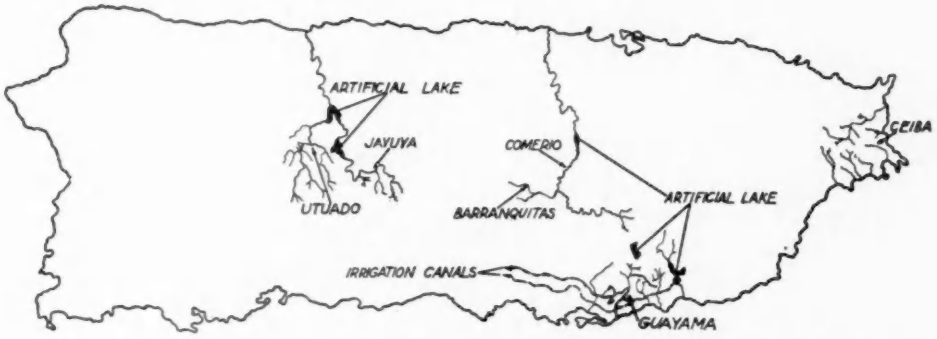


FIG. 1. Map of Puerto Rico with the 6 endemic foci of *S. mansoni* investigated, and their watersheds

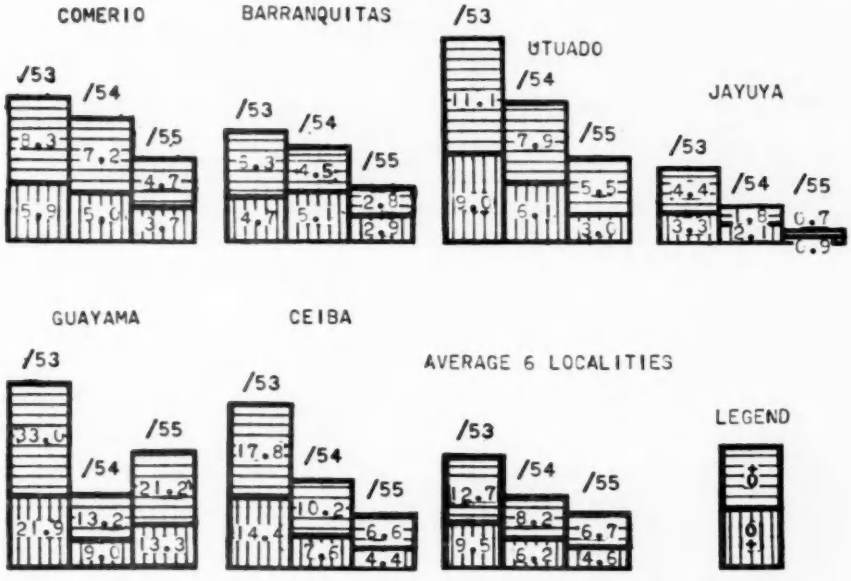


FIG. 2. Prevalence of *S. mansoni* over a 3-year period in 6 localities of Puerto Rico

calities was done by house-to-house visiting and in prenatal clinics.

*Method of fecal examination.* During the first year of the study sodium sulphate-Triton-ether centrifugation was employed as the concentration technique, complemented with one direct-saline smear. Beginning with the second year, the sodium sulphate method was replaced by formalin-Triton-ether. This technique has been observed (Maldonado *et al.*, 1954) to possess the same merits as the former for the diagnosis of schistosomiasis, while offering a better opportunity for the study of other intestinal parasites.

Egg counting was introduced during the second year also, the standardized direct-saline smear method of Beaver (1949, 1950) being adopted as the most practical one; in the meantime an evaluation of the technique was performed and the results appear elsewhere (Maldonado, 1956). Counts were made on all fecal samples positive for *S. mansoni* eggs, whether by the direct-saline preparation or upon concentration.

## RESULTS

The accompanying figures and table present the data obtained during the 3 years of investiga-

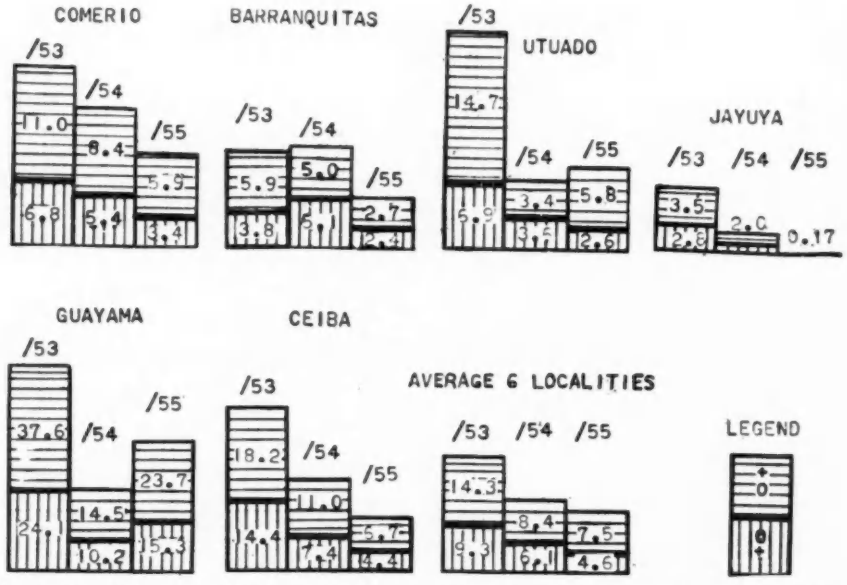


FIG. 3. Prevalence of *S. Mansoni* in individuals under 15 years of age during 3 years in 6 localities of Puerto Rico.

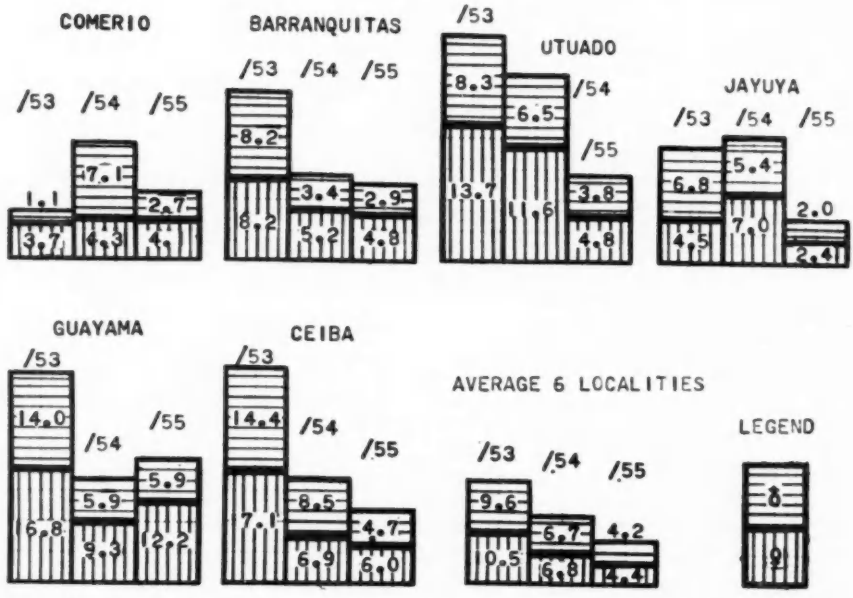


FIG. 4. Prevalence of *S. mansoni* in individuals above 15 years of age during 3 years in 6 localities of Puerto Rico.

tion. Figures 2, 3 and 4 indicate the prevalence of schistosomiasis in respect to time, place, sex, and age. In each of these figures the total prevalence at any point may be obtained by adding the percentages for males and females, given separately in each column.

It will be observed that there was a tendency for the prevalence rates to decrease over the 3-year period, dropping from 22.2 to 11.3 per cent (Fig. 2). The decrease was reflected in both young and old as evidenced by Figures 3 and 4. It may also be noted that Guayama was the only area where this trend was not consistent. Here the prevalence was higher in the third year than in the second.

The parasite was most prevalent in persons in

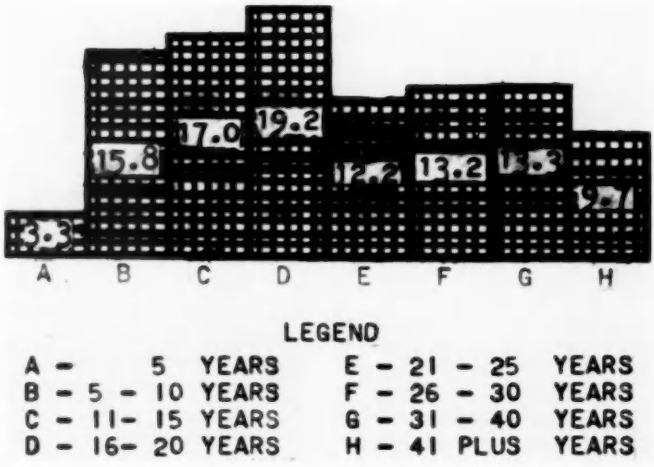


FIG. 5. Age distribution of *S. Mansoni* among individuals from 6 localities of Puerto Rico—average for 3 years.

TABLE 1

*The general prevalence of* S. mansoni *by age and by sex over a 3-year period in six different foci investigated in Puerto Rico*

| Age | 1953 | | | | 1954 | | | | 1955 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Examined | | % positive | | Examined | | % positive | | Examined | | % positive | |
| | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ |
| -5 | 79 | 71 | 6.3 | 4.2 | 30 | 30 | 6.0 | 0.0 | 26 | 35 | 3.8 | 0.0 |
| 5-10 | 1213 | 1215 | 19.4 | 29.3 | 1997 | 1821 | 11.3 | 16.4 | 1799 | 1684 | 9.8 | 15.6 |
| 11-15 | 854 | 856 | 18.7 | 30.0 | 1308 | 1248 | 12.5 | 22.1 | 1380 | 1429 | 8.5 | 15.1 |
| 16-20 | 239 | 183 | 19.7 | 33.9 | 309 | 212 | 14.9 | 32.1 | 308 | 240 | 8.1 | 16.2 |
| 21-25 | 130 | 35 | 14.7 | 25.7 | 169 | 45 | 11.8 | 6.7 | 181 | 32 | 9.4 | 12.5 |
| 26-30 | 190 | 56 | 16.3 | 42.8 | 203 | 47 | 9.3 | 17.1 | 177 | 56 | 6.8 | 3.6 |
| 31-40 | 343 | 133 | 19.9 | 30.1 | 416 | 194 | 9.3 | 17.0 | 387 | 115 | 6.4 | 13.9 |
| 41+ | 331 | 198 | 10.3 | 20.7 | 396 | 290 | 7.3 | 9.6 | 342 | 191 | 2.9 | 13.6 |
| Totals | 3399 | 2747 | | | 4828 | 3887 | | | 4600 | 3782 | | |
| Totals positive | 612 | 823 | | | 549 | 731 | | | 384 | 567 | | |
| General average (%)... | | | 18.1 | 30.0 | | | 11.4 | 18.8 | | | 8.4 | 15.0 |

the second decade of life. Although this becomes apparent by comparing Figures 3 and 4, the difference is made more evident in Figure 5, which groups the 3-year findings into various age intervals. Increasingly higher rates of infection were encountered up to 20 years of age, after which they decreased. Beyond 41 years the prevalence was only half as high as that in young adults.

Table 1 shows the relationship of sex and age to the prevalence of schistosomiasis after combining the data from the six foci. Except for the age group under 5 years, the rest of the figures show a predominance of infection in males, irrespective of age. From the general averages it may be observed that nearly twice as many men were infected as women; i.e., 20 per cent as compared with 12 per cent.

## DISCUSSION

The data in this report cannot be compared directly with previous findings, because of the use of different coprological methods and incomplete coverage or limited analysis of results by others. González-Martínez (1916), in the earliest published reports on the parasite, referred only to two areas, i.e., Utuado, studied in the present work, and Mayagüez. In 1913 that author found 220 cases of schistosomiasis in Utuado among 10,140 individuals examined, a prevalence of 2.1 per cent. Hoffman and Faust (1934), carried out the first, extensive study of the problem, although some of their data were obtained from local officials and the figures were not analyzed in detail. Weller and Dammin (1945) covered only Selective Service registrants between the ages of 18 and 38. White, *et al.*, (1957) conducted a well-organized survey among school children of municipalities not included in the present work.

It is apparent from the figures obtained by us in 1953, that the infection was significantly prevalent in the areas surveyed. A tendency towards a decrease in prevalence was observed in the following years, however.[2] It is difficult to explain this trend. Except for Guayama, where limited amounts of a molluscicide were applied outside of the irrigation canals, and in the Daguao focus of Ceiba, where a systematic therapeutic campaign was carried out among the few infected individuals, no other control measures had been practiced.

A tendency for snail populations to decrease, observed by us in the years previous to the investigation, might be offered as a possible reason for part of the change in certain areas. However, in the irrigation canals of Guayama no reduction in snail population was observed, yet schistosomiasis prevalence decreased.

Since the parasite may live in man for many years one cannot expect the overall prevalence of infection to decline markedly soon after vector populations fall. However, the incidence in small children should fall quickly.

Other factors should be sought as contributors to the apparent decrease. The marked improvement of the socio-economic status and health of the people during recent years should be emphasized in this connection. Due to these changes it is reasonable to believe that fewer children may now acquire the infection while an increasing number of adults may undergo self-cure or enter into the chronic, stool-negative phase of the disease because of lack of re-exposure.

The findings point to the importance of age in the epidemiology of schistosomiasis. The second decade of life appears to constitute the period during which persons come more often into contact with polluted waters. Upon entering adulthood, the chances of exposure decrease markedly unless parasitism is related to occupation. The rates of infection should decrease progressively from then on, with increase in age. The low prevalence recorded for the 21- to 25-year age group (Fig. 5) may be due to sampling errors since, as has been stated, the 21- to 25-year-old individuals proved the least cooperative and the ratio of female to male donors was 5:1.

The observed relation of sex to schistosomiasis is self-explanatory, inasmuch as the male leads a freer life, particularly during the first two decades of life. This predominance of schistosomiasis in the male persists up till maturity.

## SUMMARY

A total of 23,262 fecal samples were examined between 1953 and 1956 from individuals in six different localities in Puerto Rico. The areas have been recognized as foci of schistosomiasis due to *S. mansoni* for many years.

The average prevalence of infection in the six localities declined from about 22 per cent in 1953

[2] A similar reduction in the prevalence of intestinal parasites was also observed, as will be reported separately.

to 11 per cent in 1955. No definite control measures had been instituted, except for limited molluscicidal activity in Guayama, and chemotherapy among infected individuals in a small focus in another area.

Age and sex have been found important in the incidence of schistosomiasis due to *S. mansoni*. The data indicate that the greater number of cases arise between 10 and 20 years of age, and among males. Except for such places as Guayama, where the infection is an occupational hazard, it appears that the majority of infections may be acquired during the first 2 decades of life.

ACKNOWLEDGEMENTS

The constant help extended by Dr. Donald B. McMullen of the Department of Medical Zoology, U. S. Army Graduate School, Walter Reed Medical Center, Washington, D. C., is greatly appreciated.

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# STUDIES ON THE AMEBA-BACTERIA RELATIONSHIP IN AMEBIASIS

## II. Some Concepts on the Etiology of the Disease

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Microbial interrelationships are involved essentially in the etiology, pathogenesis, and pathology of intestinal amebiasis. *Entamoeba histolytica*, alone, appears incapable of producing the disease. This was suggested by data derived from previous investigations by Phillips *et al.*, (1955). These data showed that when *E. histolytica* was introduced intracecally into conventional guinea pigs, ulcerative amebiasis was produced. When the ameba was introduced into germfree guinea pigs, however, it failed either to produce disease or to survive. Hence it appeared that the etiological role of *E. histolytica* may depend upon one or more of its microbial associates in the intestine, and that amebic dysentery may be a disease of synergistic etiology, with two or more microorganisms cooperating to produce the syndrome.

More recently, investigations of this etiological phenomenon were continued. It was believed that if *E. histolytica* could be maintained in the germfree animal, more conclusive information regarding its inherent disease-producing potential might be obtained. Since the environment of the germfree intestine had appeared to lack some of the requisite factors for amebic growth, certain of the environmental characteristics of the germfree intestine were investigated. Unfortunately, very little precise information is available concerning the growth requirements of *E. histolytica*. In this connection, however, the oxidation-reduction (O-R) potentials and the pH values of the cecal lumen of germfree guinea pigs were ascertained. These were compared with values obtained in conventional guinea pigs, and evaluated with respect to their adequacy for permitting amebic growth. On the basis of these comparative results, chemical reducing agents were administered to certain germfree animals at the time of amebic inoculation.

Another area of major interest in the investigations concerned the ability of the ameba to survive within sterile tissues. In this connection, the cecal tissues of germfree guinea pigs were severely traumatized and the amebic inocula were introduced into an area adjacent to the damaged tissue. Such a procedure, it was postulated, might permit *E. histolytica* to invade the various tissue layers, and information might thus be obtained regarding the ability of the ameba to survive therein and, possibly, its pathogenic effects *per se* on such tissue.

The final area of interest in the studies was concerned primarily with the relationship of bacteria to the ameba in the etiology of intestinal amebiasis. It has appeared that bacteria may contribute factors which are essential to the establishing of amebic infection and to the pathogenesis of amebic disease. The identity of such factors, however, or of the mechanisms involved in their utilization by the ameba, remains unknown. The studies attempted to ascertain whether such factors are provided only through continuing activities of living bacteria, or whether they may be present in adequate quantity in heat-inactivated preparations of bacteria and their metabolic end-products. To obtain this information, autoclaved cecal content from conventional guinea pigs and the supernate or sediment from centrifuged preparations thereof were administered orally to germfree guinea pigs prior to, and following, the amebic inoculation. In related experiments, filtrates of cecal content and bacterial cultures were administered intracecally at the time of amebic inoculation. In some of the experiments, amebic lesions were produced in germfree animals. The results of the investigations have provided some suggestions concerning the role of bacteria in the etiology of intestinal amebiasis.

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[3] These studies were conducted at the Lobund Institute, University of Notre Dame, aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Notre Dame, NR: 131-167.

[4] Presented at the Fifth Annual Meeting of the American Society of Tropical Medicine and Hygiene, New Orleans, November 3, 1956.

## MATERIALS AND METHODS

The methodology employed in the obtaining, inoculation, examination, and determination of the microbiological status of the germfree guinea pigs was the same as that described previously by Phillips *et al.*, (1955). All amebic inocula were prepared from ameba-trypanosome cultures maintained by the method of Phillips and Bartgis (1954). This method has been demonstrated to be effective for ensuring the virulence of *E. histolytica*. The diet employed in the present studies was designated as L-445, and was composed of the following ingredients: Quaker Oats 100.0 gm, Purina Laboratory Chow (ground) 100.0 gm, dry yeast (Fleischmann's) 15.0 gm, NaCl 5.0 gm, dextrose 10.0 gm, and water 1,000 ml. This was supplemented with an aqueous vitamin preparation containing ascorbic acid, 1.0 per cent, and thiamine HCl, 0.05 per cent. The L-445 diet in refrigerator-type, ice-cube trays and the vitamin preparation in 10.0-ml glass ampoules were autoclaved in the germfree tanks at 250°F for 20 minutes. The vitamin supplement was added to the L-445 diet at one of two daily feedings at a minimum rate of 1.0 ml per animal.

Measurements of the O-R potentials in the ceca of germfree and conventional guinea pigs were obtained with a Beckman, Model G, meter equipped with platinum and standard calomel reference electrodes. Following their removal from the germfree tanks, the animals were anesthetized, laparotomies were performed, and the tips of the electrodes were inserted through small incisions of the cecal wall into the lumen. Potentiometric readings were taken immediately.

Measurements of the pH values of the cecal contents were made also with a Beckman, Model G, meter in the conventional manner. Glass and calomel electrodes were immersed in a sample of the collected cecal contents.

The chemical reducing agents employed in the experiments were sodium thioglycollate (Baltimore Biological Laboratory), and L-cysteine HCl (Nutritional Biochemical Corporation). These chemicals, in 0.4 per cent aqueous solutions, were sealed in glass ampoules and autoclaved into the germfree tank at 250°C for 20 minutes. No attempts were made to determine the effects of autoclaving on the preparations. The solutions were employed individually and were administered intracecally at the rate of 2.0 ml per animal in association with the amebic inoculum.

Trauma of the cecal tissues of certain germfree guinea pigs was induced by the following procedures: Some of the animals received 20 perforations of the cecal wall in an area approximately 20 mm in diameter surrounding the inoculation site. The perforations were made with a 23-gauge hypodermic needle. In other animals, the trauma was produced by scraping the cecal epithelium surrounding the site of inoculation with the point of a hypodermic needle. Both procedures employed needles free from amebae. Varying degrees of hemorrhage were noted in the traumatized areas of animals treated by both procedures.

The cecal material used in the experiments was obtained from adult, conventional guinea pigs. This was collected in approximately 4 parts, by volume, of physiological saline. After autoclaving at 250°F for 20 minutes the material was mixed with an equal part, by volume, of the L-445 diet at each feeding for 2 days prior to, and 2 days following, inoculation with *E. histolytica*. In one experiment, the cecal material was centrifuged for 15 minutes at 2,000 rpm prior to its entry into the germfree tank. In this instance the sediment and supernate, respectively, were fed to separate groups of germfree animals.

Other fractionations of the cecal material were obtained by its passage through Seitz filters of various known porosities. The filtrates, in most instances, were autoclaved into the germfree tanks at 250°F for 20 minutes. Other filtrates were rendered sterile by passage through filters of 0.5 $\mu$ porosity; these were handled aseptically and taken into the germfree tanks without heat treatment. All the filtrates were administered intracecally at the rate of 3 to 4 ml per animal, at the time of amebic inoculation.

In one experiment *Bacillus subtilis* was introduced into germfree animals as a contaminant of the amebic inoculum. The results were of interest and are reported herein. In related experiments, autoclaved 48-hour broth cultures of *B. subtilis* or a sterile, nonheated filtrate of similar cultures, prepared with a Seitz filter of 0.5 $\mu$ porosity, were administered to germfree guinea pigs. These preparations were given intracecally, at the rate of 3.0 ml per animal, at the time of amebic inoculation.

In other instances *E. histolytica* was introduced into the peritoneal cavity of germfree guinea pigs. In one experiment the amebic inoculum was introduced into the peritoneal cavity following laparotomy. In another experiment the amebic inoculum, accompanied by 2.0 ml of a 0.4 per cent solution of L-cysteine HCl, was injected through the abdominal wall.

## EXPERIMENTAL DATA

The results of the investigations, for the most part, are shown in Tables 1 through 5. Table 1 shows that the average O-R potential for ten germfree animals was −90.3 mv, that for ten conventional animals, −367.2 mv; a differential of 276.9 mv between the germfree and conventional animal groups, with the former having the higher potential. Similar results were obtained in germfree guinea pigs which received an inoculum of amebae-*T. cruzi*. The potentials were very high as compared to the measurements obtained for conventional animals.

On the other hand, no significant differences were noted between pH values of the cecal content of germfree guinea pigs and those of conventional guinea pigs. Values for germfree animals ranged from 6.7 to 7.5; in most instances, between 7.0 and 7.4. Values for conventional animals ranged from 6.8 to 7.8; most often around

TABLE 1

*Oxidation-reduction potentials (mv)*

A. Uninoculated animals*

| Readings | Germfree | Conventional |
|---|---|---|
| Average | −90.3 | −367.2 |
| Range | −63 to −120 | −338 to −394 |

B. Inoculated animals†

| No. | Postinoculation days | Results‡ | O-R pot. (mv) |
|---|---|---|---|
| 1 | 1/24 | P | −138 |
| 2 | 1/12 | P | −112 |
| 3 | 1/4 | P | −111 |
| 4 | 1 | P | −85 |
| 5 | 3 | N | −118 |
| 6 | 7 | N | −120 |
| 7 | 8 | N | −112 |
| 8 | 39 | LA(p) | −89 |
| 9 | 62 | LA | −96 |

* Ceca of 14-day-old, guinea pigs maintained on diet L-445.

† Ceca of germfree guinea pigs inoculated with *E. histolytica* and sacrificed at indicated intervals. Animals No. 1, 2, and 3 received no supplement to the L-445 diet. All others, except No. 4, were fed autoclaved cecal content from conventional guinea pigs for 2 preinoculation and 2 postinoculation days; No. 4 received the material for 2 preinoculation days only.

‡ See footnote to Table 4.

TABLE 2

*Results of intracecal inoculation of germfree guinea pigs with strain 200* E. histolytica *plus 2.0 ml of reducing agent**

| Animal number | Postinoculation days | Results† |
|---|---|---|
| 1 | 32 | LA(p) |
| 2 | 32 | N |
| 3 | 32 | LA(p) |
| 4 | 32 | N |
| 5 | 32 | N |
| 6 | 10 | N |
| 7 | 19 | LA(p) |
| 8 | 21 | N |
| 9 | 21 | LA |
| 10 | 21 | LA(p) |
| 11 | 21 | LA(p) |
| 12 | 21 | LA |
| 13 | 21 | N |
| 14 | 21 | N |
| 15 | 21 | LA(p) |
| 16 | 29 | LA |
| 17 | 29 | LA(p) |
| 18 | 33 | N |
| 19 | 33 | LA(p) |
| 20 | 33 | N |
| 21 | 33 | N |
| 22 | 33 | LA(p) |
| 23 | 33 | N |
| 24 | 33 | LA(p) |
| 25 | 41 | LA(p) |
| 26 | 41 | N |
| 27 | 41 | LA |

* Animals 1 to 5 received sodium thioglycollate, 0.4%; animals 6 to 27 received L-cysteine HCl, 0.4%.

† See footnote to Table 4.

7.5. Values in both germfree and conventional guinea pigs were believed to be well within a range satisfactory for growth of *E. histolytica*.

Results of amebic inoculations of germfree animals which received chemical reducing agents along with amebic inoculum are shown in Table 2. These data show that two of five animals which received sodium thioglycollate and 13 of 22 animals which received L-cysteine HCl developed amebic lesions. The lesions observed were on the cecal wall surrounding the site of inoculation. These lesions ranged from 15 to 40 mm in diameter and were characterized by a raised, whitish appearance upon gross observation. Quite often the affected area of the cecal wall was adherent to a similar lesion at the site of the incision through the abdominal wall. Scrapings of the lesions usually revealed moderate to large numbers of active *E. histolytica* but, occasionally, no amebae were demonstrable. In no instance were multiple lesions of the intestine observed. Other results, not presented in the table, showed that similar lesions did not occur either in eight germfree guinea pigs which received intracecal injections of L-cysteine HCl alone or in three animals which received intracecal injections of the reducing agent and *T. cruzi*.

TABLE 3

*Results of intracecal inoculation of germfree guinea pigs with strain 200* E. histolytica; *the ceca being traumatized at time of inoculation by the procedures indicated*

| Animal No. and traumatizing procedure | Postinoculation days | Results‡ |
|---|---|---|
| *Procedure A:** | | |
| 1 | 6 | N |
| 2 | 7 | N |
| 3 | 33 | N |
| 4 | 33 | LA(p) |
| 5 | 33 | N |
| *Procedure B:*† | | |
| 6 | 28 | N |
| 7 | 28 | LA(p) |
| 8 | 28 | LA |
| 9 | 28 | LA(p) |
| 10 | 28 | N |
| 11 | 28 | N |
| 12 | 28 | N |
| *None:* | | |
| control | 3 | P |
| control | 14 | N |
| control | 17 | N |
| control | 33 | N |

* A: Twenty perforations of cecal wall with 23-gauge hypodermic needle in an area approximately 20 mm in diameter surrounding site of inoculation.

† B: Ceca. epithelium scraped in a 20-mm-diameter area around site of inoculation with point of 23-gauge hypodermic needle.

‡ See footnote to Table 4.

The results of amebic inoculations of germfree guinea pigs which were subjected to severe trauma of the cecal wall at the time of inoculation are shown in Table 3. These data show that amebic lesions occurred within the traumatized areas of 4 of 12 germfree animals which were so treated. The lesions were similar to those which occurred in the preceding series with the chemical reducing agents. Scrapings of the lesions revealed active amebae in three of the four affected animals. There was no evidence of either old or developing lesions in other areas of the intestine. Four control animals which received amebic inoculations without additional trauma remained free from amebic lesions.

The data in Table 4 show that, of 50 germfree guinea pigs fed autoclaved cecal content from conventional guinea pigs for 2 days prior to and 2 days following amebic inoculation, 16 developed amebic lesions. Of this number, 15 animals developed only a local lesion at the site of amebic inoculation. The other animal, however, developed, in addition, a lesion approximately 12 mm in diameter which appeared not to be associated with the site of inoculation. Scrapings of the lesion revealed large numbers of active amebae. Whether the production of this lesion may have been aided by accidental damage to the cecal epithelium in the area during either inoculation or administration of the anesthetic could not be ascertained. The shortest postinoculation period wherein an amebic lesion was observed in the series was 7 days, in 2 animals; active amebae were found in both instances. The oldest lesions in the series were observed at 62 and 63 days, respectively. The lesion observed at 62 days was completely walled off and no amebae were demonstrable. The lesion observed on the sixty-third postinoculation day, however, contained numerous active amebae.

The results of a related experiment wherein germfree guinea pigs were fed either the auto-

TABLE 4

*Results of intracecal inoculation of germfree guinea pigs with strain 200* E. histolytica; *all animals fed autoclaved preparation of cecal content from conventional guinea pigs for 2 preinoculation and 2 postinoculation days*

| Animal No. | Post-inoculation days | Results* | Animal No. | Post-inoculation days | Results* |
|---|---|---|---|---|---|
| 1 | 2 | P | 26 | 31 | N |
| 2 | 2 | P | 27 | 31 | N |
| 3 | 3 | P | 28 | 31 | LA(p) |
| 4 | 3 | P | 29 | 31 | N |
| 5 | 3 | P | 30 | 32 | N |
| 6 | 4 | N | 31 | 32 | N |
| 7 | 4 | P | 32 | 37 | LA(p) |
| 8 | 6 | N | 33 | 39 | ML(p) |
| 9 | 7 | N | 34 | 40 | N |
| 10 | 7 | N | 35 | 40 | N |
| 11 | 7 | LA(p) | 36 | 40 | N |
| 12 | 7 | LA(p) | 37 | 40 | LA(p) |
| 13 | 8 | N | 38 | 40 | LA(p) |
| 14 | 8 | LA(p) | 39 | 41 | N |
| 15 | 9 | N | 40 | 41 | N |
| 16 | 9 | N | 41 | 41 | LA(p) |
| 17 | 12 | N | 42 | 41 | LA(p) |
| 18 | 13 | N | 43 | 41 | LA(p) |
| 19 | 22 | N | 44 | 41 | LA(p) |
| 20 | 23 | N | 45 | 41 | N |
| 21 | 24 | LA(p) | 46 | 43 | N |
| 22 | 26 | N | 47 | 43 | N |
| 23 | 26 | LA(p) | 48 | 62 | LA |
| 24 | 30 | N | 49 | 63 | N |
| 25 | 31 | N | 50 | 63 | LA(p) |

* N = negative for amebae and lesions. P = positive for amebae in the lumen only. LA = localized abscess at the site of inoculation; no amebae were observed in scrapings of the lesion. LA(p) = localized abscess at the site of inoculation; amebae were observed in scrapings of the lesion. ML(p) = multiple lesions or lesion not at the site of inoculation with amebae in scrapings of the lesion(s).

claved sediment or supernate from a centrifuged preparation of conventional cecal content, are as follows: Three of five animals which received the supernate, and two of five animals which received the sediment, developed localized lesions containing active amebae at the site of inoculation. These lesions were similar to those observed in the immediately preceding series.

In the next experimental series, the cecal content of conventional guinea pigs was passed through Seitz filters of various known porosities. After autoclaving, the filtrates were administered intracecally to germfree animals at the time of amebic inoculation. Twenty-nine germfree guinea pigs were inoculated in four experiments which employed filtrates passed through filters of 4–5, 5–6, 7–8, and 10 $\mu$ porosities, respectively. Ten of these animals developed a localized lesion, containing active amebae, on the cecal wall at the site of amebic inoculation. The lesions occurred in each of the four experiments and/or in the presence of each of the four filtrates. On the other hand, ten germfree guinea pigs which received a filtrate prepared with a filter of 0.5 $\mu$ porosity, and which received an amebic inoculation, remained free from amebic lesions. Ten germfree control animals which received amebic inoculation without any filtrate supplement also remained free from amebic lesions.

In two related experiments 17 germfree guinea pigs received intracecal injections, along with the amebic inoculum, of two different lots of unheated filtrates of conventional cecal content. These filtrates were bacteriologically sterile following passage of the material through filters having a porosity of 0.5 $\mu$. None of these animals developed amebic lesions. Incidentally, however, seven of nine animals which received one of the lots of unheated filtrates succumbed to acute bronchopneumonia of undetermined etiology; the other two animals showed pneumonic changes in the lungs when sacrificed.

The results of intracecal amebic inoculation of guinea pigs which received *Bacillus subtilis* as a contaminant in the amebic inoculum, are shown in Table 5. These data show that of eight animals inoculated seven developed amebic lesions. Although three of the affected animals developed only localized lesions at the site of inoculation, the other four had multiple lesions with extensive ulceration of the ceca and, in two instances, the upper colon nearest the cecum. Numerous active amebae were present in scrapings of the lesions in all seven animals. Amebae were found in the contents of the lumen in two instances.

Additional data in Table 5 show that some of the germfree guinea pigs which were given autoclaved broth cultures, or an unheated filtrate of

TABLE 5

*Results of intracecal inoculations of germfree guinea pigs with strain 200* E. histolytica

| Animal No. and group | Postinoculation days | Results§ |
|---|---|---|
| *Group A:** | | |
| 1 | 1 | P |
| 2 | 52 | ML(p) |
| 3 | 52 | LA(p) |
| 4 | 52 | LA(p) |
| 5 | 52 | LA(p) |
| 6 | 52 | ML(p) |
| 7 | 52 | N |
| 8 | 52 | ML(p) |
| 9 | 52 | ML(p) |
| *Group B:*† | | |
| 10 | 13 | N |
| 11 | 28 | LA |
| 12 | 28 | N |
| 13 | 28 | N |
| 14 | 28 | LA(p) |
| 15 | 28 | LA |
| 16 | 30 | LA(p) |
| 17 | 34 | N |
| 18 | 34 | N |
| 19 | 34 | N |
| 20 | 34 | N |
| 21 | 34 | LA(p) |
| 22 | 34 | N |
| *Group C:*‡ | | |
| 23 | 11 | N |
| 24 | 13 | LA(p) |
| 25 | 14 | N |

* Group A animals were contaminated with *Bacillus subtilis* at time of amebic inoculation.

† Group B animals received autoclaved broth culture of *B. subtilis* intracecally at time of amebic inoculation.

‡ Group C animals received unheated filtrate of a similar culture of the bacterium intracecally at time of amebic inoculation.

§ See footnote to Table 4.

cultures of *B. subtilis*, developed amebic lesions. Thirteen animals received autoclaved cultures of the bacterium along with the amebic inoculum. Five of these developed amebic lesions. Localized lesions occurred at the site of amebic inoculation and active amebae were observed in scrapings of the lesions in three of the animals. The unheated filtrate of cultures of *B. subtilis*, prepared with a Seitz filter of 0.5 $\mu$ porosity, was injected with the amebic inoculum in only three germfree animals. These three animals succumbed to intestinal paralysis. These was considerable lysis of mesenteric tissue which was believed due to a lytic factor in the filtrate. One of the animals developed a localized lesion, containing active amebae, at the site of amebic inoculation.

In another experiment, five germfree guinea pigs received intraperitoneal inoculation of *E. histolytica* in association with 2.0 ml of a 0.4 per cent solution of L-cysteine HCl. The amebae and chemical agent were injected through the abdominal wall. Three of these animals developed amebic lesions, containing active amebae, on the abdominal wall at the site of inoculation. Five germfree guinea pigs which received similar injections of L-cysteine HCl with no amebae showed no tissue changes.

In a related experiment, seven germfree guinea pigs received intraperitoneal inoculation of *E. histolytica* with no reducing agent. In this experiment, however, laparotomies were performed and the amebic inoculum was introduced directly into the exposed abdominal cavity. When the animals were sacrificed on the nineteenth postinoculation day, six had developed an amebic lesion on the abdominal wall at the site of the incision. Active amebae were found in scrapings of the affected tissue in three of the animals. There was no involvement of tissues of the intestine.

The final experiments of the series were designed to ascertain whether *E. histolytica* might be damaged during preparation of the inoculum and its introduction into the germfree tanks, to such an extent as to render it incapable of producing acute disease. To obtain this information, two germfree animals from each of three experiments were removed from the germfree tanks at 2, 4, and 24 hours, respectively, following amebic inoculation. Laparotomies again were performed immediately on each animal and 0.5 ml of a suspension of mixed bacteria was injected intracecally. One of the two animals removed from the germfree system 24 hours following amebic inoculation developed acute ulcerative amebiasis. All four animals which were removed from the tanks at lesser postinoculation intervals succumbed to the amebic disease. Eleven inoculated germfree animals which remained in the germfree units were free of amebic lesions.

During the course of the investigations the number of germfree animals available often was

inadequate to permit utilization of germfree animals as experimental controls. In this connection, however, it is pertinent to point out that, up to the present time, a total of 39 germfree guinea pigs maintained on diet L-412 (Phillips *et al.*, 1955) and 34 such animals maintained on diet L-445, have received intracecal inoculation with *E. histolytica* without any chemical or biological supplements to the amebic inoculum. None of these animals has developed amebic lesions.

## DISCUSSION

*Entamoeba histolytica* is regarded universally as the etiological agent of amebic dysentery in man, and of a similar condition which may be produced experimentally in certain laboratory animals. Data reported previously (Westphal, 1937; Phillips *et al.*, 1955; Phillips, 1957) however, and the results of the studies described herein, raise the question as to whether this ameba is the sole cause of the disease. These data suggest that bacteria may share the etiological responsibility with *E. histolytica* and that synergism of the ameba with bacteria may be prerequisite to the development of intestinal amebiasis.

Although any concepts of the role of bacteria in the etiology of the disease are at this time essentially hypothetical, one hypothesis, especially, seems worthy of consideration. It has been shown that growth of *E. histolytica* is not sustained in a variety of media wherein its growth is considerable if certain bacteria are present. Similarly, it has been shown that growth of the ameba is not sustained in the intestinal lumen of germfree guinea pigs wherein its growth is considerable if certain bacteria are present. Hence it is suggested that the role of bacteria in this disease may be, first of all, to provide within the intestine of the host an environment, physical and/or chemical, wherein the ameba may become established and grow.

This conditioning of the environment within the intestine may represent the most fundamental contribution of bacteria to the etiology of intestinal amebiasis. However, additional factors, contributed by bacteria, would appear to be involved in the acute forms of the disease. During the present investigations, *E. histolytica*, when exposed to traumatized, germfree, intestinal tissue, or when accompanied by certain chemical or biological preparations, became established at the sites of inoculation in certain tissues of germfree animals and multiplied therein for periods up to 2 months. In these instances, however, the conventional disease process did not develop. All animals were free from noticeable symptoms, and the amebic lesions were walled off by the defenses of the host. Instead of extensive ulceration of intestinal tissue, which characterizes the disease in conventional hosts, the lesions were confined either to small areas within traumatized intestinal tissue or to the areas adjacent to the sites of inoculation. Tissue damage seemed almost insignificant as compared to that which occurs in the presence of bacteria.

*E. histolytica*, when disassociated from bacteria in the intestine, has appeared to be a relatively harmless parasite. However, the seriousness of the disease it may produce in association with suitable bacteria can hardly be overestimated. Although it is not known to what extent either the ameba or bacteria participate in producing the disease, neither of these microbes, alone, appears capable of producing similar lesions. *E. histolytica* is the always-present microbial agent in this distinct disease. The same, however, may be said for bacteria.

## SUMMARY

*Entamoeba histolytica* has failed either to survive for more than a few days or to produce lesions following its introduction into the ceca of germfree guinea pigs. Investigations of certain physical characteristics of the germfree cecum provided no conclusive explanation for this phenomenon. Small, localized, amebic lesions occurred in traumatized tissues or adjacent to the sites of amebic inoculation in germfree guinea pigs which received certain chemical or biological preparations. In these instances, however, the conventional amebic disease did not develop and the lesions were confined to small areas by the defenses of the host. It was concluded that bacteria are involved essentially in the etiology of intestinal amebiasis and that synergism of ameba and bacteria is a prerequisite to development of the disease.

## ACKNOWLEDGMENTS

Collaboration of the following individuals in providing services which were essential to the investigations is gratefully acknowledged: Dr. Helmut A. Gordon; Prof. Morris Wagner; Messrs. Daniel Moran, Bernard Teah, and Erwin Zelmer;

and Miss Merrillyn Tasher, all of Lobund Institute, University of Notre Dame. Appreciation is expressed also to Mr. Samuel Poiley, Laboratory Aids Branch, National Institutes of Health, for providing a continuous supply of carefully selected gravid guinea pigs of excellent quality from which all the germfree animals were obtained by Caesarian section.

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# THE DEVELOPMENT OF A PYRIMETHAMINE-RESISTANT LINE OF *TOXOPLASMA* UNDER IN VITRO CONDITIONS[1]

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The resistance of a microorganism to a drug may be due to a natural refractoriness of a particular strain or to characteristics acquired by exposure to nonlethal concentrations of the drug. Goodwin and Rollo (1955) reported that resistance to pyrimethamine in malaria can be induced easily, generally remains a permanent character, and may be a measure of the ease with which the inhibited enzyme systems can be bypassed. Rollo (1952) reported a high degree of resistance to pyrimethamine in *Plasmodium gallinaceum* and *P. berghei*, and Schmidt and Genther (1953) and Hernandez *et al.*, (1953) reported pyrimethamine-resistant strains in *P. cynomolgi* and *P. vivax*.

The development of pyrimethamine-resistant lines of *Toxoplasma* has not been investigated previously, but may be of some importance in the treatment of clinical toxoplasmosis. The empirical regimen of treatment of human toxoplasmosis presently used is rather prolonged (2 or more weeks) and sublethal doses of the drug, as measured by anti-folic activity, may be present in the serum for at least 1 week after the end of the treatment (Goodwin, 1952). Drug-resistant lines of *Toxoplasma* may also be useful in studies of metabolic pathways present in the parasite, in cross-resistance or in genetic studies.

## MATERIALS AND METHODS

The techniques of tissue culture, drug preparation, and methods of handling the parasite were the same as those reported for tests of the in vitro effects of pyrimethamine on *Toxoplasma* (Cook and Jacobs, 1958).

In attempts to produce a pyrimethamine-resistant line of *Toxoplasma* the following procedures were used: Five tubes of monkey-kidney epithelium were infected with two million toxoplasmas (RH strain) each and pyrimethamine was added in a concentration of 0.001 mg per cent. After a 3-day incubation period in roller tubes at 37°C the cells were displaced into the supernatant medium by means of a "rubber policeman" and the cell suspensions from all five tubes were pooled. This pooled suspension of cells was centrifuged at 1,500 rpm for 2 minutes and the supernatant fluid was discarded. The sediment was resuspended in 1.5 ml of maintenance medium and then inoculated intraperitoneally into two mice. The mice were examined by paracentesis daily after the third day of infection for formation of peritoneal exudate. When fluid containing toxoplasmas was obtained, it was diluted 1:5 with maintenance medium and inoculated in 0.5-ml amounts into each of five tubes of monkey-kidney epithelium which were treated with twice the concentration of pyrimethamine contained in the previous tissue-culture tubes. This procedure was repeated through 15 transfers of pyrimethamine-treated cultures of monkey-kidney cells.

## RESULTS

Table 1 gives the history of one line, designated RES-I, in which toxoplasmas were eventually maintained in vitro in concentrations of drug up to 16.0 mg per cent. The RES-I line of the parasite was at least 640 times more resistant to pyrimethamine than the parent strain for which the minimum effective dose of pyrimethamine was 0.025 mg per cent for 3 days (Cook and Jacobs, 1958).

In the cultures treated with 0.001 and 0.002 mg per cent pyrimethamine, some degree of lysis of the cell sheets, due to proliferation of toxoplasmas, was observed by the end of the 3-day incubation period. At concentrations of pyrimethamine of 0.004 mg per cent and higher, no cytopathogenic effect was seen in cultures; the presence of parasites in these cultures was demonstrated by the inoculation into mice of the cell suspensions prepared as described above. The

[1] A portion of a dissertation submitted to the George Washington University in partial satisfaction of the requirements for the degree of Doctor of Philosophy.

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[3] Laboratory of Tropical Diseases.

TABLE 1

*Development of a pyrimethamine-resistant line of* Toxoplasma

| Culture No. | Conc. of pyrimethamine (mg %) | Survival time of mice (days) |
|---|---|---|
| RES-1 | 0.001 | 6 |
| RES-2 | 0.002 | 7 |
| RES-3 | 0.004 | 6 |
| RES-4 | 0.008 | 5 |
| RES-5 | 0.016 | 8 |
| RES-6 | 0.032 | 9 |
| RES-7 | 0.064 | 11 |
| RES-8 | 0.128 | 11 |
| RES-9 | 0.256 | 8 |
| RES-10 | 0.512 | 8 |
| RES-11 | 1.000 | 7 |
| RES-12 | 2.000 | 4 |
| RES-13 | 4.000 | 11 |
| RES-14 | 8.000 | 9 |
| RES-14.5 | 12.000 | 7 |
| RES-15 | 16.000 | 7 |

variations in survival times of the mice thus inoculated probably are indicative of differences in the number of organisms that survived the various concentrations of drug.

Several experiments were performed to determine the stability of the drug-resistant line, RES-I, in the absence of pyrimethamine. Cultures of RES-I were maintained by biweekly passages in untreated tissue cultures of monkey-kidney cells for a period of 3 months. At monthly intervals the parasites in these cultures were tested for their ability to survive in the presence of 16.0 mg per cent pyrimethamine. Because of the toxic effects of pyrimethamine-lactic acid solution on the cells in these cultures, the observation period was necessarily short, only 7 days. During this period no lysis of the cultures occurred but the cell suspensions from them were infective to mice. Therefore, it appears that the pyrimethamine-resistance of this line of *Toxoplasma* was a relatively stable acquired characteristic.

The RES-I parasites maintained without drug for 3 months proliferated rapidly, as judged by the need for biweekly passages. This in itself is evidence that, although drug-resistance was established, the parasites had not developed a dependency on the drug during the period of adaptation. However, since microorganisms frequently develop a requirement for a drug when cultivated continuously in the presence of sublethal concentrations (Hinshelwood, 1952), further tests of this possibility were performed. Cultures of the RES-I line of *Toxoplasma* were grown for a period of 3 months through tissue cultures of monkey-kidney cells containing 1 or 5 mg per cent pyrimethamine. When the organisms harvested from these cultures were transferred to cultures containing no pyrimethamine, there was no indication that drug-dependency had developed; these organisms produced lysis as rapidly as those from cultures not maintained in the presence of the drug.

In addition to tests of the RES-I line in tissue cultures, two experiments were performed to test the RES-I line in vivo. Drug diets were prepared by adding pyrimethamine to powdered mouse diet and by subsequent blending in a Hobart Mixer for 5 minutes. The mice in these experiments weighed between 14 and 17 grams, and were started on the drug diets on day zero of the infection. Survivors were maintained on these diets for a period of 3 weeks, after which they were given normal laboratory chow and observed for 3 additional weeks. Five mice were used for each drug group and each mouse received an inoculum of 20,000 toxoplasmas intraperitoneally. Table 2 gives the results of one of these in vivo tests.

Mice that received the lower concentrations of pyrimethamine, up to 15 mg per cent, in the diet, all died of toxoplasmosis. The only evidence of the effect of the drug was a prolongation of

TABLE 2

*Comparison of the mouse-passed line and the RES-I line of the RH strain of* Toxoplasma *for resistance to pyrimethamine in vivo*

| Pyrimethamine conc. in diet (mg %) | No. of mice dead of 5 inoculated with 20,000 parasites | |
|---|---|---|
| | RH | RES-I |
| 0.0 | 5 (9.6)* | 5 (9.8) |
| 5.0 | 5 (10.2) | 5 (10.3) |
| 10.0 | 5 (12.0) | 5 (11.6) |
| 15.0 | 5 (16.3) | 5 (13.2) |
| 20.0 | 2 (18.5) | 2 (29.0) |
| 25.0 | 0 | 0 |

* Figures in parentheses are the average survival times of the mice (days).

the survival time of the treated mice, as compared to the controls. Here, the increase in survival time was practically the same for the RES-I line and its parent RH strain. At the higher dosages of pyrimethamine the same number of mice survived RH and RES-I infections; the differences in survival time of those mice that died despite administration of 20 mg per cent pyrimethamine are probably of no significance. Dye tests performed on the serums of the survivors were uniformly negative, indicating cures of the animals infected with both the RH and RES-I lines. Results similar to these were obtained in replicate in vivo tests. It is therefore apparent that the RES-I line did not manifest any resistance to pyrimethamine in mice comparable to its ability to survive the drug in tissue culture.

## DISCUSSION

From the type of data obtained in the tests reported here, it is not possible to say whether *Toxoplasma* developed increased resistance to pyrimethamine because of contact with the drug or whether the resistant nature of the RES-I line was due to an artificial selection of a mutant that developed in the early transfers. It also is not possible to say definitely that the resistant parasites were capable of proliferating to any great extent in the presence of high concentrations of pyrimethamine. It can be stated, however, that these organisms maintained their ability to invade cells and to persist in these cells in the presence of concentrations of the drug many times higher than that effective in clearing nonresistant toxoplasmas from tissue cultures in a 3-day exposure period.

The failure to demonstrate drug-resistance in vivo in lines of *Toxoplasma* able to survive in high concentrations of pyrimethamine in vitro possibly is explainable, not so much by assuming growth and adaptation of the organism in the presence of the drug, as by the hypothesis that these parasites survive exposure to drug because they are in a dormant state. The latter has been suggested by the occasional finding of positive cultures after treatment with high concentrations of pyrimethamine in the tests reported by Cook and Jacobs (1958) on the concentrations of pyrimethamine in tissue culture.

## SUMMARY

1. A pyrimethamine-resistant line of *Toxoplasma* at least 640 times more resistant than the parent strain was developed in vitro.

2. The resistant nature of this line was retained for at least 3 months when the parasite was grown in the absence of pyrimethamine.

3. A pyrimethamine-requiring line of *Toxoplasma* could not be developed.

4. The drug-resistant nature of this line of *Toxoplasma* was not demonstrable in vivo.

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# DENGUE ANTIBODIES IN RESIDENTS OF THE SOCIETY ISLANDS, FRENCH OCEANIA

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Although it has been known since World War II that there are at least two distinct immunologic types of dengue virus (Sabin, 1952), relatively little data are available on the geographic distribution of each of these types and on their respective importance in the etiology of endemic and epidemic dengue. One of the reasons for this paucity of information is that dengue often occurs in areas in which other group-B arthropod-borne viruses (Casals and Brown, 1954) are endemic. Antibody studies in such areas are difficult to interpret because of the broad immunologic response which occurs in persons who have been infected with two or more viruses of this group (Casals, 1957).

In 1944, an extensive epidemic of dengue occurred in the Society Islands (Rosen, *et al.*, 1954), a relatively remote oceanic area in which no other group-B arthropod-borne viruses are known to occur. Data will be presented in this report on the dengue neutralizing and hemagglutination-inhibition antibodies present in sera collected from residents of this area in 1951 and 1952.

## MATERIALS AND METHODS

*Sera.* The 87 sera employed were part of a larger group collected in 1951 and 1952 in connection with studies on poliomyelitis (Horstmann and Kraft, 1955; Kessel, *et al.*, 1956).[1] The number of sera studied from each island was as follows: Tahiti, 3; Moorea, 32; Raiatea, 7; Huahine, 27; and Maupiti, 18. Specimens were selected for this study, and for the various tests within the study, solely on the basis of quantities of serum available. The present tests were carried out in 1956 after the sera had been stored at either refrigerator (4°C) or deep freeze (−20°C) temperature for 4 to 5 years.

*Viruses.* The mouse-adapted Hawaiian strain of dengue type 1, the mouse-adapted New Guinea "C" strain of dengue type 2, and a recently isolated strain of yellow fever from Trinidad were employed.

*Serologic procedures.* Neutralization (NT) and hemagglutination-inhibition (HI) tests were carried out and interpreted by methods which are described in detail elsewhere (Rosen, 1958a).

In brief, neutralization tests were performed in suckling mice using a single test dosage calculated to contain 100 $LD_{50}$ of virus. The actual dosages employed as determined by titrations in the individual tests were 300, 300, and 500 for the three dengue 1 tests, and 60, 80, and 300 for the three dengue 2 tests. Sera were not diluted or inactivated. Serum-virus mixtures were incubated for 2 hours at 37°C before inoculation.

Hemagglutination-inhibition tests were carried out in plastic plates using 16 units of hemagglutinin prepared by the alkaline-buffer extraction method of Sweet and Sabin (1954). Normal inhibitor was removed from serum by acetone extraction.

## RESULTS

Since only small amounts of many of the sera were available, it was not possible to test all sera for both NT and HI antibodies against the two dengue viruses. Twenty-seven sera were tested for dengue 1 and/or dengue 2 NT antibodies and 72 sera (including 12 of those tested for NT antibodies) were screened for HI antibodies against both viruses. No differences were noted in analyzing the results by individual island and hence the entire group of sera will be considered as a whole.

*Neutralizing antibodies.* Of 27 sera tested for dengue 1 NT antibodies, 25 (93%) were positive and two were negative. Twenty-one of the dengue 1 positive sera were also tested for dengue 2 NT antibodies with the following results: 2 positive, 5 inconclusive, and 14 negative. Both of the persons positive for dengue 2 NT antibodies were more than 50 years of age. One of the dengue 1 negative sera was also tested against dengue 2

[1] These sera were kindly made available by Dr. J. F. Kessel of the University of California at Los Angeles.

with a negative result. The distribution of dengue 1 NT antibodies by age group is shown in Table 1.

*Hemagglutination-inhibition antibodies.* Of the 72 sera screened at a 1:10 dilution in the HI test against the two dengue antigens, 64 (89%) were positive against dengue 1 and 61 (85%) were positive against dengue 2. No sera were positive against dengue 2 alone. The distribution of dengue 1 HI antibodies by age group is also shown in Table 1. It will be noted that no positive sera were found among six children less than 7 years of age (i.e., born after 1944); whereas, each of the nine children 7 to 9 years old was positive.

A total of 42 HI-positive sera from among those screened was titrated against both dengue antigens. In 31 instances the dengue 1 titer was higher than that of dengue 2, in nine instances the titers were the same, and in two instances the dengue 2 titer was the higher. A frequency distribution of the respective titers against the two dengue antigens is shown in Table 2.

Fifteen HI-positive sera were also titrated against a yellow fever antigen as well as against both dengue antigens. The titers for individual sera against all three antigens are shown in Table 3. It will be noted that only three sera (all with high dengue HI titers) had yellow fever HI antibodies.

TABLE 1

*Dengue 1 neutralizing (NT) and hemagglutination-inhibition (HI) antibodies in residents of the Society Islands, by age group*

| Age group (years) | Antibodies | | |
|---|---|---|---|
| | NT | HI | NT and/or HI |
| 1–6 | 0/1* | 0/6 | 0/7 |
| 7–9 | 1/1 | 9/9 | 10/10 |
| 10–19 | 9/9 | 19/20 | 25/26 |
| 20–29 | 5/5 | 11/11 | 15/15 |
| 30–39 | 4/5 | 9/10 | 10/11 |
| 40+ | 6/6 | 16/16 | 18/18 |
| Total | 25/27 | 64/72 | 78/87 |

* Number of persons positive/Number of persons tested.

TABLE 2

*Frequency distribution of dengue 1 and 2 HI titers for 42 sera from residents of the Society Islands*

| HI titer | Number of sera with indicated titer | |
|---|---|---|
| | Dengue 1 | Dengue 2 |
| 320 | 1 | 0 |
| 160 | 11 | 4 |
| 80 | 19 | 8 |
| 40 | 7 | 15 |
| 20 | 3 | 12 |
| 10 | 1 | 3 |
| Total | 42 | 42 |

TABLE 3

*Dengue and yellow fever HI titers for 15 sera from residents of the Society Islands*

| Serum No. | Titer | | |
|---|---|---|---|
| | Dengue 1 | Dengue 2 | Yellow Fever |
| 1 | 320 | 40 | 0 |
| 2 | 160 | 80 | 40 |
| 3 | 160 | 80 | 10 |
| 4 | 160 | 80 | 10 |
| 5 | 160 | 40 | 0 |
| 6 | 160 | 40 | 0 |
| 7 | 80 | 80 | 0 |
| 8 | 80 | 40 | 0 |
| 9 | 80 | 40 | 0 |
| 10 | 80 | 40 | 0 |
| 11 | 80 | 40 | 0 |
| 12 | 80 | 20 | 0 |
| 13 | 80 | 20 | 0 |
| 14 | 40 | 40 | 0 |
| 15 | 40 | 20 | 0 |

## DISCUSSION

Although the sera studied were few in number, the results seem to indicate: 1) that dengue 1 is the only type of dengue virus that has been active in the Society Islands in recent years, 2) that infection with this virus had been very prevalent and widespread, and 3) that the virus apparently disappeared after the 1944 epidemic.

These serologic data are in accord with clinical observations in that no cases of dengue were observed in the area in either natives or newly arrived persons from 1944 to 1952 (Rosen, 1952, unpublished data). The explosive nature of previous epidemics, which were characterized in such terms as, "few inhabitants escaped" and "not a

native was spared" (Mumford and Mohr, 1944), also suggested that the disease was not endemic there. It is of interest to note that dengue virus disappeared in spite of the continued presence of a suitable, abundant, and ubiquitous vector, *Aedes polynesiensis* (Rosen, *et al.*, 1954). Apparently no mechanism exists in the Society Islands for the maintenance of dengue virus after the rapid exhaustion of the human nonimmune population.

The neutralization of dengue 2 virus by two sera could be interpreted either as a cross-reaction resulting from a dengue 1 infection or, perhaps, in view of the age of the persons from whom the sera were obtained, an earlier occurrence of this type of dengue in the area.

The dengue HI titers of positive sera from the present study were remarkably similar to those of monkeys which had been experimentally infected with dengue 1 virus (Rosen, 1958b). In both instances relatively high heterologous HI titers against dengue 2 virus were present and very few sera had HI antibodies against dengue 1 virus alone. In contrast, both natural infections in humans (Rosen, 1958a) and experimental infections in monkeys (Rosen, 1958b) with dengue 2 virus give rise to much lower, and often no, heterologous HI titers against dengue 1 virus.

## SUMMARY

The examination of sera from residents of the Society Islands in French Oceania for dengue-neutralizing and hemagglutination-inhibition antibodies indicated that: 1) dengue 1 virus is the only type of dengue virus that has been active in the area in recent years, 2) infection with this virus had been very prevalent and widespread, and 3) the virus apparently disappeared after 1944 (when the last epidemic of the disease occurred).

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# EXPERIMENTAL INFECTION OF NEW WORLD MONKEYS WITH DENGUE AND YELLOW FEVER VIRUSES[1]

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It has long been suspected that monkeys might play a role in the epidemiology of dengue (Simmons, *et al.*, 1931). However, only two genera of Old World monkeys (*Macaca* and *Cercopithecus*) and the chimpanzee are known to be susceptible to infection with this virus (Sabin, 1952), and no information has been available on the susceptibility of any of the monkeys which occur in the New World.

In the course of studies on the epidemiology of dengue in Panamá (Rosen, 1958a), six genera of of New World monkeys were inoculated with unmodified strains of dengue virus. These experiments were undertaken to obtain information on the potential role of these animals as natural hosts of dengue virus, and on their suitability as experimental animals for laboratory studies. In addition, their serologic responses were studied in order to obtain data which might aid in interpreting the significance of dengue hemagglutination-inhibition (HI) antibodies in man. In connection with this latter objective, additional monkeys of some of the genera were inoculated with a recently isolated strain of yellow fever virus and with the 17D strain of this virus.

## MATERIALS AND METHODS

*Monkeys.* All the New World monkeys used in the present experiments were captured in the Republic of Panamá. Prior to their use, all were screened for dengue 1 and 2 and yellow fever HI antibodies and none was found. Specimens of the following weights and species were employed: the white face monkey (*Cebus capucinus*), 800 to 1,900 gm; the red spider monkey (*Ateles geoffroyi*), 3,300 gm; the black spider monkey (*Ateles fusciceps*), 1,400 to 5,300 gm; the howler monkey (*Alouatta palliata*), 1,350 to 3,400 gm; the marmoset (*Marikina geoffroyi*), 400 to 600 gm; the Saimiri monkey (*Saimiri örstedii*), 450 to 600 gm; and the night monkey (*Aotus trivirgatus*), 500 to 750 gm. All animals were housed in a doubly screened room for at least 30 days following their viral inoculations. The larger species of monkeys were kept in outdoor cages at other times.

*Virus strains for monkey inoculations.* For the dengue experiments, sera from persons experimentally infected with strains of each type of dengue virus (Hawaiian strain of type 1 and New Guinea "C" strain of type 2) which had never been passaged in mice were used as inocula.[2] For yellow fever a recently isolated strain of yellow fever virus from Trinidad (in the second and third mouse brain passages), and the 17D strain of this virus from a commercial lot of vaccine, were employed.

*Serologic procedures.* Neutralization (NT) and HI tests were carried out and interpreted by methods which are described in detail elsewhere (Rosen, 1958a). In brief, neutralization tests for dengue were performed in suckling mice using a single test dosage calculated to contain 100 $LD_{50}$ of virus. The sera were not inactivated and serum-virus mixtures were incubated for 2 hours at 37°C before inoculation. HI tests were carried out in plastic plates using 16 units of hemagglutinin prepared by the alkaline buffer extraction method of Sweet and Sabin (1954). Normal inhibitor was removed from serum by acetone extraction.

*Tests for unmodified dengue virus.* Serum to be tested for virus was separated from the clot within 2 hours of the time that it was drawn and was then stored in sealed glass ampoules in a chest containing solid $CO_2$ until tested (about 1 year later). Each serum dilution to be tested was inoculated intracerebrally in 0.01 to 0.02 ml amounts into two litters of 1-day-old suckling mice of the National Institutes of Health general-purpose white Swiss strain. The mice were then observed for 28 days. Those mice which survived

[1] Most of the work herein reported was carried out while the author was assigned to the Gorgas Memorial Laboratory, Panamá, Republic of Panamá.

[2] These sera were kindly supplied by Dr. A. B. Sabin, Children's Hospital Research Foundation, Cincinnati, Ohio.

for this period of time were then challenged with 1,000 $LD_{50}$ of mouse-adapted dengue virus of the same type as that suspected to be present in the serum being tested. The mice were then observed for an additional 21 days. Mice which died within 7 days of the original inoculation or within 5 days of the challenge inoculation were considered to have died from extraneous causes and were excluded from the calculations. Mice which died between 8 and 28 days following the original inoculation or which survived the challenge inoculation were considered to have been infected with dengue virus from the original inoculum.

## RESULTS

*Dengue.* Thirty monkeys were given a single inoculation subcutaneously or intraperitoneally of from 0.1 to 0.5 ml of undiluted human serum containing either type 1 or type 2 dengue virus. Although none of the animals subsequently showed overt signs of illness or died, 24 of them apparently became infected as indicated by the appearance of HI and/or NT antibodies. All monkeys were tested for dengue 1 and 2 and yellow fever HI antibodies following inoculation and all of those which showed no HI antibodies (as well as a few of those which did) were then tested for dengue NT antibodies.

The number of monkeys of each species inoculated with each type of dengue virus and the number which developed HI or NT antibodies are shown in Table 1. It will be noted that, with one exception, at least one monkey of each species developed antibodies after inoculation with each type of dengue virus. Both of the monkeys which failed to develop antibodies after injection with type 1 dengue were inoculated with serum from the same vial (three of five monkeys inoculated with this serum developed antibodies). Likewise, each of the four monkeys which failed to develop antibodies after injection with type 2 dengue were inoculated with serum from another vial (three of seven monkeys inoculated with this serum developed antibodies.)

TABLE 1

*Number of monkeys inoculated with either dengue 1 or 2 viruses and numbers which developed HI or NT antibodies to the type virus inoculated, by species*

| Species of monkey | Dengue 1 | Dengue 2 |
|---|---|---|
| *Cebus capucinus* | 3/5* | 5/6 |
| *Ateles geoffroyi* | 1/1 | 1/1 |
| *Ateles fusciceps* | 1/1 | 2/3 |
| *Alouatta palliata* | 1/1 | 0/1 |
| *Marikina geoffroyi* | 2/2 | 1/1 |
| *Saimiri örstedii* | 2/2 | 2/2 |
| *Aotus trivirgatus* | 2/2 | 1/2 |
| Total | 12/14 | 12/16 |

* Number which developed HI or NT antibodies/number inoculated.

TABLE 2

*Dengue infections in suckling mice inoculated with sera of experimentally infected monkeys*

| Source of serum | Dilution tested | Mouse mortality ratio; original inoc. | Mouse survival ratio; challenge inoc. | Mouse infection ratio |
|---|---|---|---|---|
| BS 166 five days post injection with dengue 1 | undiluted | 4/7† | 3/3‡ | 7/7§ |
| | 1:10 | 5/14 | 5/9 | 10/14 |
| WF 320 five days post injection with dengue 1 | 1:10 | 3/12 | 4/9 | 7/12 |
| | 1:100 | 0/16 | 1/16 | 1/16 |
| Controls* dengue 1 | undiluted, 1:10, or 1:100 | 3/36 | 1/33 | 4/36 |
| RS 88 five days post injection with dengue 2 | undiluted | 0/4 | 4/4 | 4/4 |
| | 1:10 | 0/14 | 6/12 | 6/12 |
| Controls* dengue 2 | undiluted or 1:10 | 0/9 | 0/8 | 0/8 |

* See text.

† Number of mice dying/number inoculated.

‡ Number of mice surviving/number challenged with 1000 $LD_{50}$ of homologous virus.

§ Number of mice infected/number tested.

*Viremia.* Four monkeys were tested for viremia. Two marmosets (M 396 and M 412) were inoculated subcutaneously with 0.5 ml of fresh whole blood taken from a howler monkey (H 379) 4 and 6 days, respectively, after inoculation with dengue 1 virus. Both marmosets subsequently developed dengue HI antibodies characteristic of dengue 1 infections (see below).

Sera obtained 5 days after inoculation from three other monkeys, two inoculated with type 1 dengue (black spider 166 and white face 320), and one inoculated with type 2 dengue (red spider 88),

were tested for viremia by the inoculation and subsequent challenge of suckling mice. This procedure was necessary because unmodified strains of dengue virus are often not lethal for mice.

The results of the mouse inoculation and challenge experiments are shown in Table 2. The "control" mice were inoculated with sera obtained from the same monkeys 2 days after injection. Although the number of mice used was small, it appeared that the sera of all three monkeys had enough dengue virus 5 days after infection to infect about one-half the mice when tested in a dilution of 1:10. Some of the mice which died following the original inoculation of serum suspected of containing dengue 1 virus were paralyzed before dying. None of the three mice which died following the inoculation of the "control" sera was paralyzed before death, and hence it was not possible to determine whether these mice died as a result of infection with small amounts of dengue virus which might have been present in these sera or whether they died from extraneous causes.

It will be noted that none of the ten mice infected[3] with unmodified dengue 2 virus from monkeys in these experiments died, whereas 12 of the 25 mice infected with unmodified dengue 1 virus from monkeys did succumb. It was found that undiluted sera from white face monkeys were lethal for mice and hence sera from this species could only be tested in a dilution of 1:10 or higher.

*Hemagglutination-inhibition antibodies.* The dengue and yellow fever HI antibody titers observed in all monkeys inoculated with human sera and the two marmosets inoculated with blood from the howler monkey are shown in Tables 3 and 4. Two monkeys inoculated with human sera containing dengue 1 virus failed to develop HI antibodies, although they did develop substantial titers of NT antibodies. Both of these animals were inoculated from the same vial of serum that failed to infect two other monkeys (see above). All monkeys which developed HI antibodies and which were tested for NT antibodies gave positive results.

It will be noted that monkeys injected with dengue 1 developed relatively high titers of HI antibodies against dengue 2. On the other hand, monkeys injected with dengue 2 developed much lower heterologous titers against dengue 1 (5 of 12 had no dengue 1 HI antibodies). It is also of interest to note that monkeys injected with dengue 2 developed heterologous titers against yellow fever equal to those against dengue 1.

TABLE 3

*Dengue and yellow fever HI antibodies in monkeys following injection with unmodified dengue 1 virus*

| Source of serum* | Days after inoculation serum obtained | HI titer | | |
|---|---|---|---|---|
| | | Dengue 1 | Dengue 2 | Yellow fever |
| M 355 | 103 | ≥640 | 320 | 160 |
| NM 382 | 111 | ≥640 | 320 | 80 |
| M 356 | 102 | 320 | 160 | 40 |
| M 412 | 88 | 160 | 80 | 10 |
| WF 439 | 69 | 160 | 40 | 20 |
| NM 383 | 111 | 80 | 80 | 10 |
| H 379 | 28 | 80 | 40 | 20 |
| BS 166 | 158 | 80 | 40 | 10 |
| RS 227 | 93 | 80 | 40 | 10 |
| M 396 | 80 | 40 | 20 | 0 |
| WF 320 | 227 | 10 | 10 | 0 |
| WF 321 | 185 | 10 | 10 | 0 |
| S 423 | 25 | 0 | 0 | 0 |
| S 425 | 25 | 0 | 0 | 0 |

* M = marmoset, NM = night monkey, WF = white face monkey, H = howler monkey, BS = black spider monkey, RS = red spider monkey, S = Saimiri monkey.

*Yellow fever.* Three marmosets and two white face monkeys were inoculated subcutaneously or intraperitoneally with 0.07 to 0.15 ml of a 10 per cent mouse brain suspension of the Trinidad strain of yellow fever. All three of the marmosets appeared ill 4 days after inoculation and one of them died 2 days later. Neither of the white face monkeys became ill. The dengue and yellow fever HI antibody titers of the four monkeys which survived and of three rhesus monkeys (*Macaca mulatta*), which were also inoculated with the same strain of virus by Dr. E. Rodaniche of the Gorgas Memorial Laboratory, are shown in Table 5.

Three marmosets and four black spider monkeys were inoculated subcutaneously or intraperitoneally with 0.5 ml of 17D yellow fever vaccine. None of the monkeys developed any illness. The dengue and yellow fever HI antibody

[3] As demonstrated by their resistance to challenge with mouse-adapted virus.

responses of these monkeys are also shown in Table 5. It will be noted that one black spider monkey developed dengue as well as yellow fever HI antibodies following inoculation with 17D virus whereas the other three monkeys of this species failed to develop even yellow fever HI antibodies. None of the four monkeys had yellow fever NT antibodies prior to inoculation but all developed substantial titers afterwards.

It appears from the limited amount of data available that the homologous HI antibody response was higher following infection with the Trinidad strain of yellow fever virus than with the 17D strain. Only one of seven monkeys inoculated with the 17D strain developed dengue HI antibodies, whereas six of seven monkeys inoculated with the Trinidad strain did so. It is of interest that the heterologous antibodies against dengue 2 were usually higher than against dengue 1.

TABLE 4

*Dengue and yellow fever HI antibodies in monkeys following injection with unmodified dengue 2 virus*

| Source of serum | Days after inoculation serum obtained | HI titer | | |
|---|---|---|---|---|
| | | Dengue 1 | Dengue 2 | Yellow fever |
| BS 186 | 94 | 40 | 320 | 40 |
| M 353 | 30 | 40 | 160 | 40 |
| S 429 | 39 | 20 | 160 | 40 |
| BS 193 | 134 | 20 | 160 | 20 |
| RS 88 | 186 | 20 | 160 | 10 |
| NM 389 | 111 | 20 | 160 | 10 |
| WF 315 | 227 | 0 | 160 | 0 |
| S 427 | 32 | 10 | 80 | 10 |
| WF 437 | 111 | 0 | 80 | 0 |
| WF 440 | 111 | 0 | 80 | 0 |
| WF 405 | 111 | 0 | 40 | 0 |
| WF 404 | 69 | 0 | 40 | 0 |

TABLE 5

*Dengue and yellow fever HI antibodies in monkeys following injection with either of two strains of yellow fever virus*

| Source of serum | Yellow fever strain | Days after inoculation serum obtained | HI titers | | |
|---|---|---|---|---|---|
| | | | Dengue 1 | Dengue 2 | Yellow fever |
| RH *237 | Trinidad | 231 | 20 | 80 | 320 |
| RH 287 | Trinidad | 192 | 20 | 80 | 320 |
| RH 65 | Trinidad | 208 | 20 | 40 | 320 |
| M 350 | Trinidad | 100 | 40 | 80 | 160 |
| BS 390 | 17D | 133 | 10 | 40 | 80 |
| M 344 | Trinidad | 58 | 10 | 0 | 40 |
| WF 361 | Trinidad | 134 | 0 | 10 | 20 |
| WF 357 | Trinidad | 92 | 0 | 0 | 10 |
| M 340 | 17D | 75 | 0 | 0 | 10 |
| M 341 | 17D | 33 | 0 | —† | 10 |
| M 343 | 17D | 75 | 0 | — | 10 |
| BS 391 | 17D | 22 | 0 | 0 | 0 |
| BS 392 | 17D | 29 | 0 | 0 | 0 |
| BS 393 | 17D | 133 | 0 | 0 | 0 |

* RH = rhesus monkey.
† = not tested.

## DISCUSSION

Although the amount of dengue virus in the human sera at the time that they were used to inoculate the monkeys was unknown and none of the monkeys became ill, the available data do suggest that most, if not all, of the animals which developed dengue HI or NT antibodies actually were infected. Thus, the dengue HI titers of most of the monkeys were equal to or higher than those of the four animals in which virus multiplication was shown to have taken place by the demonstration of a viremia 4 to 6 days after inoculation. Also, the dengue HI titers of most of the animals were similar to those which occur following natural (Rosen, 1958a and b) or experimental (Sweet and Sabin, 1954) dengue infections in man and experimental infections in monkeys known to be susceptible to these viruses (Rosen, *et al.*, 1954). Finally, in the instance in which only three of seven monkeys inoculated with one lot of type 2 dengue virus developed HI or NT antibodies (which suggested that only a very small amount of virus was present in the inoculum) the animals which did develop HI antibodies (BS 186, BS 193 and NM 389) had relatively high titers as compared to the completely negative results with sera of the other four animals. Similarly, in the instance in which only three of five monkeys inoculated with one lot of type 1 dengue virus developed HI or NT antibodies, the monkey which did develop HI antibodies (WF 439) had relatively high titers. The sera of two monkeys of this group which developed only NT antibodies gave a clear-cut positive result in the neutralization test as compared with clear-cut negative

results (with no prolongation of the average survival time of the mice) for sera from the other two monkeys.

If the HI and NT antibodies observed in the experiments cited above had been the result only of the antigenic stimulus of virus in the inoculum, with no multiplication in the monkeys, one would expect a gradation of antibody responses among the various animals rather than the clear-cut, strongly positive and completely negative results which were observed.

Among the monkeys used in the present experiments are representatives of both families, Callitrichidae and Cebidae, of monkeys which occur in the New World. In view of the data which have been presented, it would seem logical to suspect that most, if not all, species of monkeys which occur in the New World are susceptible to infection with dengue viruses.

It is of interest to note that in common with the other monkeys known to be susceptible to infection with dengue viruses and the chimpanzee (Sabin, 1952), none of the New World monkeys showed any overt clinical response to infection.

The amounts of data available from the current experiments on viremia are insufficient to warrant conclusions regarding ability of the New World monkeys to serve as sources of infection for mosquitoes. It should be noted, however, that other species of monkeys have been used successfully for this purpose in the laboratory (Simmons, *et al.*, 1931; Rosen, *et al.*, 1954).

It is perhaps significant that none of the 43 monkeys used in the present experiments, or of 62 other monkeys of the same species also captured in Panamá, was found to have naturally-occurring antibodies that could be attributed to dengue infections (Rosen, 1958a). Although New World monkeys are apparently susceptible to infection with the dengue viruses, there is as yet no evidence that they are exposed to, or acquire, these viruses under natural conditions (Rosen, 1958a).

The HI antibody titers noted in the monkeys infected with dengue 1 and 2 viruses are strikingly similar to those which have been observed in persons naturally infected with these two types of virus (Rosen, 1958a and b). Apparently dengue 1 virus produces a broader antigenic response to dengue 2 virus than the latter does to dengue 1 virus. It is remarkable that dengue 2 virus produces just as high HI titers against yellow fever virus as against dengue 1 virus.

As might be expected, the HI antibody responses to infection with a virulent strain of yellow fever virus were greater than those which followed injection with the 17D strain of this virus. The relatively meager dengue HI antibody response to infection with 17D virus was similar to that which has been noted in man (Rosen, 1958a).

## SUMMARY

Thirty monkeys of six genera which occur in the New World were inoculated with strains of type 1 and type 2 dengue virus which had never been passaged in laboratory animals. Although none of the monkeys showed overt signs of illness, almost all developed hemagglutination-inhibition (HI) or neutralizing antibodies. Viremia was demonstrated in four species of monkeys of three different genera 4 to 6 days after inoculation.

The dengue and yellow fever HI antibody responses of these monkeys and others inoculated with yellow fever virus were similar to those which have been observed in persons naturally infected with these viruses.

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# ECOLOGICAL STUDIES OF WILD RODENT PLAGUE IN THE SAN FRANCISCO BAY AREA OF CALIFORNIA

## II. Efficiency of Bacterial Culture Compared to Animal Inoculation as Methods for Detecting *Pasteurella pestis* in Wild Rodent Fleas[1]

S. F. QUAN, H. von FINTEL and A. G. McMANUS

An enzootic plague focus, similar to the limited foci described by Meyer *et al.*, (1943) and Baltazard *et al.*, (1952) was studied in the San Francisco Bay region (Kartman *et al.*, 1958). During an epizootic in this focus in March–April 1954 the weekly plague infection rate of the fleas from wild rodents was as high as 10.8 per cent and averaged 6.6 per cent for 4 weeks.

Usually, *Pasteurella pestis* infection of fleas collected from nature is extremely low (Pollitzer, 1952) and the determination of infection rates by culturing the fleas individually is tedious and time-consuming. The procedure generally used and recommended is the inoculation of susceptible laboratory animals with aqueous suspensions of pooled flea triturates (Eskey and Haas, 1940; Baltazard *et al.*, 1956). The comparative efficiency of these two methods for detecting *P. pestis* has not been evaluated in the case of naturally infected fleas. Various flea species collected during this epizootic provided an adequate source of naturally infected individuals to evaluate and compare the efficiencies of these two methods for detecting *P. pestis*. The results are presented here.

### MATERIAL AND METHODS

*Source of fleas.* Fleas[2] were collected from live-trapped rodents in a sylvatic plague focus in the San Bruno Mountain area of the San Francisco Bay region of California. They were brought into the laboratory in vials containing aqueous 2 per cent sodium chloride and 0.01 per cent "Tween 80." These vials were stored at 4°–10°C in a refrigerator for periods of from overnight to 5 days before the fleas were removed from the saline solution, identified, and placed singly in small test tubes (13 × 100 mm).

*Bacterial culture.* A sterile glass rod (3 × 150 to 200 mm), the ends of which were roughened, was dipped about one cm into a sterile aqueous suspension of 1 per cent "Attasorb"[3] and used to crush and triturate a flea. To each flea triturate then was added 0.2 ml of sterile distilled water and a suspension was made by stirring with the rod. The rod then was removed, and the suspension adhering to it was streaked onto a section of a blood agar plate. The plates were incubated at 28°C and examined after 24 and 48 hours. Usually the agar plates were observed for a total of 5 days. All bacterial colonies resembling those of *P. pestis* were examined with a stereoscope, and whenever necessary were tested for staining and biochemical reactions, for lysis with specific bacteriophage (Gunnison *et al.*, 1951; Cavanaugh and Quan, 1953), and for pathogenicity by animal inoculation.

*Animal inoculation.* For routine detection of plague, fleas are generally pooled for animal inoculation (Swellengrebel and Hoesen, 1915; Pollitzer, 1952). To pool the suspensions of fleas remaining after bacterial culture, from one to several tubes of flea triturates were washed into a sterile mortar with 1 to 2 ml of physiologic saline solution. Whenever necessary, the resulting pool was triturated again. Then the suspension was aspirated into a syringe and inoculated subcutaneously into either a mouse or a guinea pig. Princeton strain laboratory mice were used primarily in this study because these have been shown to be very susceptible to experimental plague infection and to inoculation of *P. pestis* toxins (Holdenried and Quan, 1956). The animals were observed for 10 to 14 days after inoculation, then sacrificed and autopsied. All plague findings were confirmed by the usual procedures (Pollitzer, 1936, 1952).

Animal inoculations were deferred 24 hours after bacterial culture of the flea. During that

[1] From the Communicable Disease Center, San Francisco Field Station, San Francisco 18, California.

[2] Mr. Virgil I. Miles and Mr. Alva Kinney supplied the fleas, and Mr. Frank M. Prince and Mr. Harold Stark identified them.

[3] A complex hydrated magnesium silicate supplied by Attapulgas Mineral and Chemical Corp., Philadelphia 5, Penn.

TABLE 1

*Fleas found infected with* P. pestis *by bacterial culture and by animal inoculation*

| Week ending | Cultured for *P. pestis* | | | | Inoculated into animals | | | | Total number fleas pos. |
|---|---|---|---|---|---|---|---|---|---|
| | No. fleas | No. positive | Pos. fleas missed* | | Flea pools | | Pos. fleas missed* | | |
| | | | No. | Per cent | No. | Positive† | No. | Per cent | |
| 3–24 | 239 | 17 | 8 | 32 | 58 | 17 | 2 | 8 | 25 |
| 3–31 | 447 | 40 | 6 | 13 | 88 | 9 | 1 | 2 | 46 |
| 4–8 | 357 | 13 | 4 | 24 | 91 | 10 | 7 | 41 | 17 |
| 4–14 | 254 | 8 | 3 | 27 | 82 | 10 | 1 | 9 | 11 |
| Totals..... | 1297 | 78 | 21 | 21 | 319 | 46 | 11 | 11 | 99 |

* Fleas harboring *P. pestis* missed by this procedure but found by the other procedure.

† Some positive pools of fleas contained more than one flea harboring *P. pestis*, as indicated by bacterial culture of individual fleas.

time, the triturates were stored in the refrigerator. The delay allowed segregation of tubes shown by culture to contain fleas presumptively positive for *P. pestis* and/or to be grossly contaminated with other bacteria.

## RESULTS

Table 1 shows the results of 4 weeks of testing. A total of 1297 fleas were cultured individually, and inoculated as 319 pools into laboratory mice. By bacterial culture 78 fleas were proved to harbor *P. pestis*, while by animal inoculation at least 21 others were proved positive in the pools. Assuming that only one positive flea was present in each of the 21 *P. pestis*-positive pools, a total of at least 99 fleas or 7.6 per cent of the fleas tested harbored plague bacilli.

Based on 99 positives the range of the efficiency for finding plague bacilli in fleas by animal inoculation varied from 59 to 98 per cent, and the average was 89 per cent. The range for the culturing method varied from 68 to 87 per cent, and the average was 79 per cent. Thus the two ranges, although unequal, have about the same midpoint, but the average for the animal inoculation procedure is skewed toward the higher values.

Further quantitative evidence of the efficiency of these methods to detect *P. pestis* in the flea at various magnitudes of infection could be obtained if the actual degrees of infection were known. For the 21 fleas found positive only by animal inoculation, the number of plague bacilli present was unknown. On the other hand, the magnitude of infection in the 11 fleas missed by animal inoculation could be evaluated from growth in the culture medium. Of these 11 fleas, four yielded less than 20 *P. pestis* colonies; three, from 20 to 100 colonies; and the other four showed innumerable colonies to confluent growth. Data to be presented in another paper of this series (Quan, Miles and Prince, unpublished) show that, during the same 4-week period concerned here, all told, 11 fleas were found by culture to yield less than 20 colonies, and eight fleas, from 20 to 100 colonies. Thus, at low infection levels, the animal inoculation procedure was effective in finding 12 positives

TABLE 2

*Fleas found harboring* P. pestis *by species,* by bacterial culture, and by animal inoculation*

| Week ending | Total positives† /by bacterial culture/by animal inoculation | | | | |
|---|---|---|---|---|---|
| | M. tel. | H. lin. | A. mul. | C. wym. | O. nes. |
| 3/24 | 18/15/16 | 5/2/5 | 0 | 0 | 2/0/2 |
| 3/31 | 33/32/33 | 10/8/9 | 1/0/1 | 1/0/1 | 1/0/1 |
| 4/8 | 11/8/6 | 6/5/4 | 0 | 0 | 0 |
| 4/14 | 8/5/7 | 2/2/2 | 1/1/1 | 0 | 0 |
| Total... | 70/60/62 | 23/17/20 | 2/1/2 | 1/0/1 | 3/0/3 |

* M. tel. = *Malaraeus telchinum;* H. lin. = *Hystrichopsylla linsdalei;* A. mul. = *Atyphloceras multidentatus multidentatus;* C. wym. = *Catallagia wymani;* O. nes. = *Opisodasys keeni nesiotus.*

† Minimum total number of fleas harboring *P. pestis*, assuming that only one positive flea was present in each pool found positive by animal inoculation, but not found by culture. It was assumed that no positive flea was missed by the combination of both procedures.

among 19 fleas; an efficiency of 63 per cent. At higher infection levels, 55 positives were determined among 59 fleas, an efficiency of 93 per cent.

In Table 2 the fleas found harboring *P. pestis* are tabulated according to species and by method of detecting infection. Of a possible 70 *Malaraeus telchinum* proved positive, ten (14%) were missed by bacterial culture, and eight (11%) were undetected by animal inoculation; of 23 *Hystrichopsylla linsdalei*, six (26%) were missed by culture; three (13%) by inoculation. Of two *Atyphloceras multidentatus*, one was missed by culture, and none was missed by inoculation. The other four positives, one *Catallagia wymani* and three *Opisodasys keeni nesiotus*, were found only by animal inoculation. Of the 21 positive fleas missed by culture, ten (47%) were *M. telchinum*; six, (29%) were *H. linsdalei*; three (14%) were *O. k. nesiotus*; and one each, or 5 per cent each, was *A. multidentatus* and *C. wymani*. Of the 11 positives missed by animal inoculation, eight (73%) were *M. telchinum*, and the remaining three (27%) were *H. linsdalei*.

The efficiencies for detecting *P. pestis* in *M. telchinum* by culture and by animal inoculation were 87 and 89 per cent, respectively; and the efficiencies for *H. linsdalei* were 74 and 87 per cent, respectively. For *A. multidentatus*, *C. wymani*, and *O. k. nesiotus*, the efficiencies by culture were 50, 0, and 0 per cent, respectively, while the success by animal inoculation was 100 per cent for each species. On the basis of the present data both methods were about equally effective for *M. telchinum*. For *H. linsdalei*, both methods were also about equally good, except in the first week when culture was poor. During that first week the culture procedure and equipment were new, and the difficulties in grinding large fleas like *H. linsdalei* were not sufficiently realized. It is also possible that other flea species presented similar problems of technique. With *A. multidentatus* a problem similar to that found with *H. linsdalei* may have existed; with *O. k. nesiotus* the fleas were apparently too tough to triturate; and with *C. wymani* the small size made grinding difficult. Nevertheless, too few fleas of these last three species were proved to have *P. pestis* to substantiate the presumed difficulties.

Initially an assumption was made that the combined use of culture and inoculation of animals would find all the fleas infected with *P. pestis*; 99 positive fleas were found in a total of 1,297 tested. Nonetheless, 99 was merely the best first estimate for evaluation of the efficiencies of the methods. With the efficiency of the detecting methods evaluated it was then possible to estimate the number of positive fleas that may have been missed. Applying the extreme ranges of the efficiency of these methods it was calculated that 0.25 to 13 per cent, or from 0 to 13 positive fleas may have been missed. Applying the average efficiencies, 79 and 89 per cent, the percentage missed was 2.3, or about 2 fleas.

TABLE 3

*Advantages and disadvantages of the culture and animal-inoculation methods for detecting* P. pestis *in fleas*

| Subject | Bacterial culture | Animal inoculation |
|---|---|---|
| Simplicity of method | Simple | Very simple |
| Simplicity of equipment | Very simple | Simple |
| Pooling fleas for test | Difficult | Simple |
| Time for results | One day | 3 to 10 days |
| Animal facilities needed | None | Some |
| Processing large numbers individually | Possible | Impractical |
| Efficiency | Very good | Excellent |
| Sensitivity | Excellent | Excellent |
| Determination of infection rate | Simple | Difficult |

## DISCUSSION

The method of inoculating animals with a suspension of flea triturate to test for the presence of *P. pestis* has enjoyed long and popular use, mainly by virtue of the fact that fleas can be pooled to reduce a large number of specimens to few inoculations (Pollitzer, 1952; Baltazard *et al.*, 1956). Table 3 shows that it is a very simple method which requires simple equipment. When guinea pigs, white mice, or other susceptible animals are used, the method is sensitive and, as shown in this report, it is highly efficient. The results of the inoculations, however, usually are not available for from 3 to 10 days, and in addition to laboratory facilities, quarters for housing infected animals are necessary. This requirement limits the number of pooled inoculations which can be processed, and the individual inoculation of large numbers of fleas is prohibitive. Besides,

certain factors may interfere with the test; such as individual resistance of the test animal to the infection, the infecting and killing of the animal by some other disease agent in the flea, and the presence of heavy bacterial contamination which may either cause the animal to die nonspecifically or abort the plague infection. Recently, some investigators (Brygoo, 1956) have partially circumvented some of these difficulties by inoculating penicillin with the flea triturates. Nevertheless, penicillin should be judiciously used since it is known that high concentrations of it in vitro will kill *P. pestis* (Meyer *et al.*, 1952).

Factors favoring use of the culture method for detecting *P. pestis* in fleas are: most of the positive results are available in about 18 hours; animal facilities are not required; when necessary, as in the evaluation of infection rates, large numbers of fleas can be individually tested. Also, as in the inoculation of animals, this method requires simple procedures and equipment. Thus the practicability of culturing *P. pestis* on a blood agar medium is excellent and the average efficiency for finding this bacterium in fleas is high, but it is generally less than that of animal inoculation (see above, and Tiflov, 1946). By using better grinding equipment (*e.g.*, a tissue homogenizer) it was possible to increase the effectiveness of culturing (Quan, Ransom, and Omi, unpublished).

The main drawback of the culture method is that the fleas could not be pooled for testing because of bacterial and fungal contaminants on, and in, the fleas. Even when the fleas were individually cultured, a few specimens harbored contaminants that either would spread and outgrow or would inhibit *P. pestis*. Over 50 per cent of the fleas tested here, however, had a very slight rate of contamination, with 5 or less colonies of bacteria; about 15 to 20 per cent had a heavy rate, with 20 to innumerable colonies. In spite of this contamination, some workers (Bichkov and Borzenkov, 1929;) have reported limited success in culture of individuals and pools of fleas for finding *P. pestis*. Others (Pollitzer, 1936, Webster, 1932) overcame the problem of contamination by dissection of the fleas and culturing only pools of proventriculi and ventriculi. These methods are too complicated and elaborate for most experimental routines.

It should be mentioned that *P. pestis* isolated from individual fleas by culture has not been tested for virulence. This may not be absolutely necessary since *P. pestis* can be readily identified with in vitro tests such as specific bacteriophage (Gunnison *et al.*, 1951; Cavanaugh and Quan, 1953) and fluorescein-antibody staining (Winter and Moody, 1957); and because avirulent plague bacilli have not yet been found in fleas.

SUMMARY

Studies with 1,297 wild rodent fleas, cultured individually and inoculated as 319 pools into animals, showed at least 99 to contain *Pasteurella pestis*. The efficiency ranges of the two methods were 68 to 87 per cent for culture, and 59 to 98 per cent for animal inoculation but the averages were 79 and 89, respectively. The efficiency of the culturing method appeared to be the same for various concentrations of *P. pestis* in the flea. Success of the animal inoculation, however, varied with the different densities of plague bacilli; it was about 60 per cent when the number of bacteria per flea was low, and over 90 per cent when the number was very high.

Both methods are simple in procedure and equipment, and both are highly sensitive in detecting the presence of plague bacilli in fleas, such as *Malaraeus telchinum* and *Hystrichopsylla linsdalei*. The culture method is rapid, it does not require animal facilities, and it allows the processing of large numbers of fleas individually. The animal inoculation of flea pools has few interfering factors, it has a higher degree of efficiency than the culturing method, and it allows the pooling of large numbers of fleas for few injections. Because *P. pestis* occurs in fleas in nature so rarely, the method of choice for surveillance remains that of animal inoculation. On the other hand, for critical study of epizootic plague among wild rodents and their fleas, individual culturing of fleas provides invaluable epizootiological data.

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# CLINICAL TRIALS OF COMPOUND 11925C[1]

## A New Amebicidal Drug

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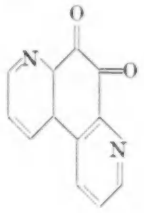
Owing to the variation among strains of *Entamoeba histolytica* and the association of infection by this parasite with bacterial infection of the intestinal mucosa, no single and absolutely safe curative amebicidal drug has been found up to the present. A new compound 11925C[1] has been synthesized which was found in vitro to be similar in its action on *E. histolytica* to emetine and only inferior to fumagillin (Kradolfer and Neipp, 1958). The drug is a phenanthroline derivative with the composition 5,6-quinone, 4,7-phenanthroline and with the structural formula:




It is slightly soluble in water, and is excreted in bile and urine. The $LD_{50}$ dose in rats is 16 times the effective dose (Kradolfer and Neipp, 1958). The manufacturers claim a 100 per cent clearance of amebas on the fifth day in infected rats on a daily dose of 50 mg/kg of body weight (Kradolfer and Neipp, 1958). According to these authors bacteriostatic action of the drug has also been demonstrated against gram-positive and gram-negative organisms (*Escherichia coli*, *Shigella*, *Streptococcus faecalis* and *Staphylococcus aureus*). Such a combination of amebicidal and bacteriostatic actions in one drug would be ideal in the treatment of amebic dysentery.

Trials with this drug against *E. histolytica* in human patients were therefore performed by us to assess its value in this respect.

### CHOICE OF PATIENTS

The study was made on 24 patients varying in age from 1 to 60 years. The patients chosen showed good numbers of the parasite in their stools. Cases with light infection were omitted to permit a fair contrast between the stool findings before and after treatment. Patients were divided, according to their signs and symptoms at the time of the first examination, into five groups as shown in Table 1.

Group I comprised 15 patients with acute dysenteric symptoms manifested with diarrhea 3 to 20 times per day, tenesmus and passage of blood and mucus.

Group II was composed of three patients complaining of abdominal colicky pains with past histories of repeated attacks of diarrhea and dysentery.

Group III included two patients complaining of pain in the right hypochondrium and past histories of repeated attacks of diarrhea. The liver was palpable in one case and tenderness over the right hypochondrium was found in both.

Group IV included one patient with no complaint but in whom, on examination, there was found a tenderness over the right hypochondrium.

Group V included three symptomless cases with no abdominal signs, the parasite being found in their stools on routine examination.

Microscopic findings are shown in Table 1.

### ADMINISTRATION OF THE DRUG

The drug was given by mouth in 7-day courses in three categories of daily doses per kg of body weight as follows:

Dose I: 4 to 5.5 mg/kg

Dose II: 5.6 to 8 mg/kg

Dose III: 8.1 to 12.5 mg/kg

Follow-up stool examinations were performed on the third, seventh, fifteenth and twenty-first days after start of treatment. No other drugs were administered.

### RESULTS

Among three patients who received Dose I, two harboured *E. histolytica* cysts and one *E. histo-*

[1] Entobex® CIBA.

TABLE 1

| Cases | Symptoms | Microscopic findings | Ages (years) | | | | Daily dose (mg/kg) | | | Cases neg. on day | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1–5 | 6–10 | 11–20 | over 20 | 4–5.5 | 5.6–8 | 8.1–12.5 | 3 | 7 | 15 | 21 |
| 15 | Acute dysentery, tenesmus, diarrhea 3–4 times and up to 20 times per day with blood & mucus | Numerous trophozoites and cysts of *E. hist.* and *Trich. intest.*; *Giardia* cysts | 10 | | 2 | 3 | 1 | 9 | 5 | 11 | 15 | 15 | 15 |
| 3 | Abdominal colicky pains & past history of repeated attacks of diarrhea | Some *E. hist.*, trophozoites; cysts of *E. hist.* and *Giardia* | | 2 | | 1 | 2 | | 1 | 2* | 2 | 2† | 2 |
| 2 | Pain & tenderness over right hypochondrium & past history of rep. attacks of diarrhea | Precysts and cysts of *E. hist.* | | | 1 | 1 | | 2 | | 0 | 2 | 2 | 2 |
| 1 | No complaint, had tender right hypochondrium | *E. hist.* cysts | | | 1 | | | | 1 | 1 | 1 | 1 | 1 |
| 3 | No complaint, no signs | *E. hist.*, trophozoites and cysts | | | 3 | | | 1 | 2 | 1 | 3 | 3 | 3 |
| Total 24 | | | 10 | 2 | 7 | 5 | 3 | 12 | 9 | 15 | 23 | 23 | 23 |

* 0 = cast still positive.
† = case not followed up.

*lytica* trophozoites. One of the first two patients became negative on the third day, the second being cleared on the seventh day. No further follow-up examinations were made of this case.

Twelve patients received Dose II, nine harboring trophozoites, the rest showing only cysts. Of the nine patients six became negative on the third day, the other three being cleared only on the seventh day. Of the three cases harboring cysts one became negative after the third day; the remaining two after the seventh day.

Nine patients, six with trophozoites, were given Dose III. Five were cured on the third day; the rest on the seventh. Three patients showing cysts were cleared of the infection on the third day.

No toxic manifestations were observed apart from the occurrence of two cases of diarrhea after the fourth day of administration of the drug, in spite of the negative findings in the stools. The diarrhea ceased spontaneously in one case and on administration of sulphaguanidine in another.

It was also observed that the drug could not clear either *Giardia intestinalis* or *Trichomonas hominis* infections which occurred concomitantly with *E. histolytica*.

Three patients had tenderness in the right hypochondrium and in one, an adult 40 years old, the liver was palpable. X-ray examination of this patient showed normal diaphragmatic movements, the right dome of the diaphragm being of normal height. The patient had a past history of repeated attacks of diarrhea, and stool examination revealed cystic and precystic forms of *E. histolytica*. The spleen was not palpable and there was no past history of intestinal schistosomiasis or jaundice and the patient did not consume alcohol. The case was assumed to be one of early amebic hepatitis. The patient received Dose II for 7 days and the stools became negative on the seventh day but the pain and tenderness over the liver persisted and there

was no retrogression in the size of the liver. The course of treatment was repeated after 2 weeks with no improvement in the liver condition.

One of the remaining two patients received Dose II and the tenderness over the right hypochondrium disappeared. The latter two cases most probably were not cases of hepatitis, the tenderness being due to lesions in the hepatic flexure of the colon.

## CONCLUSIONS

The drug described herein was given orally to 24 patients infected with *Entamoeba histolytica*, in 7 day courses in three categories of daily doses; 4 to 5.5, 5.6 to 8 and 8.1 to 12.5 mg/kg of body weight with the following results:

1) The drug was effective in the three dosages used; the smaller doses requiring a longer treatment for clearing the infection.

2) Best results were obtained using 8.1 to 12.5 mg/kg of body weight daily, clearance of infection being obtained in eight cases of nine on the third day and in all on the seventh day.

3) The drug seems to be ineffective in treating amebic hepatitis.

4) The drug failed to cure *Giardia* or *Trichomonas* infections.

5) No toxic symptoms appeared apart from mild diarrhea in two patients after the fourth day of starting treatment. One of these was cured spontaneously and the other by administration of sulphaguanidine.

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# THE PERSISTENCE OF ACQUIRED IMMUNITY TO THE FILARIAL WORM OF THE COTTON RAT[1]

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We have shown in a previous report (Macdonald and Scott, 1953) that when cotton rats are infected with the filarial worm, *Litomosoides carinii*, and then reinfected after about 30 days, the worms of the second infection are retarded in growth as compared with those of parallel primary infections in similar animals. We have called this retardation of growth an expression of immunity since it apparently is good evidence of a power to resist development of infection arising in response to a previous infection. Some authors have referred to responses occurring under similar conditions as "immunity to superimposed infection." Premunition is another term which might be applicable. This term was introduced by Sergent *et al.*, (1924) for use in discussing malaria and other protozoal infections. Later, in an approved English translation, they defined premunition as applying to "Chronic diseases giving a good resistance only as long as the chronic infection is lasting" (Sergent *et al.*, 1925). This definition raises the question which formed the basis of the present experiments, namely whether the phenomenon we have observed persists after the original infection dies out. The question is especially pertinent in view of facts we have reported (Scott *et al.*, 1958a; 1958b) which lead to the conclusion that the retardation of growth is induced principally during the first 7 days of the primary infection. How long, then, does this "immunity" persist in the absence of reinfection?

## METHODS AND MATERIALS

The infections were all administered by the methods described by Scott and Macdonald (1953). Infective larvae were dissected from mites which had been allowed to feed once on an infected cotton rat. The larvae were accumulated in a drop of half-strength Tyrode's solution on a cover glass where they were examined for the removal of immature and damaged specimens. The rest were counted and poured from the cover glass into a subcutaneous pocket made by surgical incision in the skin of the rat. The challenging or test infections were given to the previously infected rats and to parallel uninfected control rats on the same day, using larvae dissected from the same lot of mites. The animals were killed 24 days later, and the worms were washed from the abdominal and pleural cavities in salt solution. The worms of the test infection were fixed, mounted, and measured as described by Westbrook and Scott (1955).

Larvae from the same lot of mites were used for all the test infections within each protocol. The parallel immunized and control rats were in several cases litter mates, or from successive litters of the same pairs, and the others were closely related animals of the same inbred strain. With the one exception noted, the experimental animals were all given a single immunizing infection of the size indicated.

## RESULTS

The detailed results of the experiments are shown in Table 1. All of the immunized rats had dead worms of the immunizing infection present at autopsy. In only 2 cases could the number present be estimated since they were so involved in tissue reactions. The first five animals listed had live worms of the immunizing infection present at autopsy in the numbers indicated. The blood of the first four of these animals was positive for microfilariae up to 5 months before the test infection. After this time no further examination of the blood was made until shortly before the test infection, when it was negative in all cases. The fifth animal had microfilariae in the blood at the time of the test infection. The last three immunized animals listed had no live worms of the immunizing infection at autopsy. The first two of these had microfilariae in the blood up to 5 months before the test infection and were

[1] Supported in part by Research Grant E 64 from the National Institutes of Health, U. S. Public Health Service.

TABLE 1

*Length of worms in test infections given 12 to 22 months after primary infections and percentage which had at least started the fourth molt on the 24th day as compared with worms of parallel primary infections**

| Protocol | Age of rats in months at time of | | Immunizing larvae given | Adult worms recovered | | Test worms | | Mean length of test worms† | | Per cent at least starting to molt | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Immunization | Test infection | | Alive | Dead | Number given | Per cent recovered | Males | Females | Males | Females |
| A | 11 | 26 | 285‡ | 39 | 6 | 74 | 30 | 38 | 44 | 0 | 0 |
| A | 4 | 23 | 191 | 3 | 40 | 72 | 25 | 41 | 54 | 0 | 0 |
| B | 5 | 22 | 150 | 6 | + | 86 | 50 | 44 | 52 | 0 | 0 |
| B | 6 | 18 | § | 199 | + | 129 | 20 | 27 | 40 | 0 | 0 |
| B | 2 | 14 | 171 | 79 | + | 104 | 36 | 40 | 52 | 11 | 6 |
| B | 2 | 16 | 111 | 0 | + | 114 | 25 | 44 | 52 | 21 | 10 |
| B | 2 | 23 | 79 | 0 | + | 134 | 49 | 76 | 112 | 96 | 41 |
| B | 3 | 25 | 132 | 0 | + | 123 | 37 | 50 | 59 | 0 | 0 |
| A | — | 25 | Control | 0 | 0 | 79 | 24 | 50 | 87 | 57 | 0 |
| B | — | 22 | Control | 0 | 0 | 102 | 35 | 74 | 106 | 88 | 16 |
| B | — | 26 | Control | 0 | 0 | 80 | 36 | 68 | 93 | 50 | 0 |
| B | — | 26 | Control | 0 | 0 | 130 | 43 | 77 | 118 | 54 | 11 |
| B | — | 26 | Control | 0 | 0 | 108 | 36 | 67 | 98 | 84 | 20 |

* Each line represents one cotton rat.

† Lengths are given in tenths of millimeters.

‡ Given 4 equal infections at 10 to 12 months of age.

§ Infected by allowing infected mites to feed for 24 hours.

negative just prior to the test infection. The last one listed never showed microfilariae in the blood on any of numerous single-drop examinations.

Two criteria were used for evaluating the differences between the rate of growth and development of the test worms in the immunized rats and those in the control rats, viz., the mean length of the worms of each sex on the 24th day, and the percentage of worms of each sex which had at least started the fourth molt by this time. It will be noted in the table that, with one exception, the mean length of the worms from the immunized rats was always less than the mean length of the worms of the corresponding sex from the control rats of the same protocol. The rat which gave the exceptional results had been given only 79 immunizing larvae, while all of the others had been given over 100 larvae. It is questionable whether the discrepancy should be attributed to this difference. Perhaps it should be attributed to random variability since we have occasionally seen discrepancies of this order in short-term immunity experiments even though all animals were treated alike. When the lengths of all the worms of each sex from all the immunized rats were pooled and compared by means of the "t" test with similar pooled values for all control rats, the differences for both sexes were statistically significant ($P = <.001$) in spite of the inclusion of the rat with divergent values.

The percentage of male worms which had at least started to molt was likewise smaller for all immunized rats, with the same exception, than for the control rats. When values were similarly pooled, the results showed that 20 per cent of the male worms from the immunized rats had reached this stage as compared with 61 per cent of those from the control rats. This difference was also statistically significant when subjected to the chi square test ($P = <.001$). It is not surprising that the female worms showed no such differences since they generally molt later than the males even though they grow more rapidly. Only 14 per cent of the females from immunized rats and 11 per cent from control rats had reached this stage and the numbers were thus too small for valid comparison.

## DISCUSSION

It seems clear that, as a general rule, the immunity persists for a long time even after the worms of the primary infection are dead. Since we are dealing with a very low-level immunity, and since we have previously noted considerable

variability in the response of individual animals, we need not be surprised that there was one exception to the rule. We appear to have obtained a satisfactory answer to our original question, but the results have raised numerous other questions.

Since the degree of immunity observed in these experiments was equal to that which we regularly find about a month after the establishment of the primary infection, the question is raised as to whether the immunity persisted for 1 or 2 years without reinforcement. One possible source of reinforcement, viz., accidental reinfection, can be eliminated. The rats were housed for the entire period in a room kept free of mites. Numerous control animals housed in the same room were autopsied from time to time to demonstrate that no accidental infection ever occurred. Another possible source would be antigens produced by the mature worms throughout their life. Previous experiments (Scott *et al.*, 1958a; 1958b) have shown that larvae in the late third stage, in the fourth stage, or in the early fifth stage have some immunizing effect although not nearly as much as an equal number of infective larvae. It is possible, then, that after they have matured they continue to have sufficient effect to maintain this immunity.

On the other hand, the immunity in some of the animals of the present experiments persisted for some time after all of the immunizing worms were presumably dead. In an earlier experiment dead adult worms introduced surgically, and live adult worms which were similarly introduced but which died immediately after introduction, had a barely measurable immunizing effect (Macdonald and Scott, 1955). A few unpublished experiments, however, showed a lack of immunizing effect of freshly ground worms injected subcutaneously with or without adjuvants. It would seem prudent to leave open the question as to whether deposits of dead worm tissue continue to have an antigenic effect until more critical experiments can be done.

The term "premunition" according to its original definition, does not seem to be a good term to apply to this situation since the immunity persists after the infection has discontinued. The term "immunity" to superimposed infection is somewhat ambiguous. The introduction of the secondary infection after the primary infection has disappeared does not seem to be quite the same thing as superimposing the secondary infection on the primary infection while the latter is still active. Perhaps some new term is needed for helminth infections, since these differ from infections with unicellular organisms; at least in the quantity of potentially antigenic material which persists long after the organisms die. Postmunition might serve as a term to express resistance (using this word in the sense of Sergent *et al.*) persisting after the termination of infection but only as long as material from the infection is present. The etymology of premunition as explained by the authors cited seems to be slightly confusing, but the term has been accepted. Postmunition would have a similar derivation from the Latin words *munire* and *post*, to fortify afterward. It might appear that this situation does not differ appreciably from cases in which dead material, or live but noninfective material, is injected in the process known as immunization. The difference would depend on the initial induction, by infection, of a resistance which is prolonged by the presence of dead or dying material from the infection.

SUMMARY

These experiments show that the retarding effect on growth and development of secondary infections of *Litomosoides carinii* in cotton rats, produced by a pre-existing primary infection, persists for 1 or 2 years after the introduction of the primary infection. It also persists for a considerable time after the death of the primary infecting worms. It is possible that this immunity, established at the time of introduction of the primary infection, persists for these long periods without reinforcement. On the other hand it seems possible, even in face of some evidence to the contrary, that the adult worms and, later, the deposits of dead worm tissue, may continue to exert an antigenic effect. Postmunition is suggested as a term which might be appropriate in a situation such as this.

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# ELIMINATION OF *LEPTOSPIRA BALLUM* FROM A COLONY OF SWISS ALBINO MICE BY USE OF CHLORTETRACYCLINE HYDROCHLORIDE

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The presence of *Leptospira ballum* in the breeding colony of Swiss albino mice at the Rocky Mountain Laboratory was first recognized in 1949. This leptospira did not cause detectable illness in mice, but unusual results of certain experimental studies led to discovery of the organism. Since husbandry methods did not preclude spread of the organism to succeeding generations of breeding stock, the disease persisted in the colony. Upon recent recognition that laboratory workers had contracted leptospirosis from these mice (Stoenner and Maclean, 1958), means of eliminating this health hazard without discarding the colony were investigated.

In preliminary epizootiologic studies (Stoenner, 1957), *L. ballum* was isolated from a high percentage of mature breeding stock (see Table 1). Antibodies against *L. ballum* were detected in serums of mice of all ages, but leptospires could be recovered only from mature mice. Progeny of seropositive females possessed antibody at birth and acquired additional quantities through ingestion of colostrum. Antibodies in these mice could still be detected at 51 days of age but not at 65 days. When progeny of seropositive females were isolated from their mothers at 21 days of age, they remained free of leptospires despite previous continuous exposure during the nursing period.

In view of the previous findings, the nucleus of a leptospira-free colony could be established by determining the serologic status of breeding females just prior to parturition and then by segregating the young from seropositive females at 21 days of age. Obviously, progeny of females seronegative at parturiton could not be included, because such dams might be in the incubative stage of the disease and they might subsequently transmit leptospires without having provided any humoral protection during gestation or in their colostrum. The preceding method of starting a new colony was not feasible because adequate space necessary to insure absolute isolation of a leptospira-free colony from the infected colony was not available.

An alternate approach of treating the colony with an antibiotic appeared to be the most practical means of eliminating the organism. Previous studies have shown that chlortetracycline eliminated leptospires from the kidneys of hamsters and swine (Brunner and Meyer, 1950; Howarth, 1956). Obviously, to eliminate *L. ballum* from the colony, every mouse had to receive a dosage sufficient to insure destruction of all organisms. If a small percentage of treated mice retained a few leptospires, such mice would soon infect succeeding generations reared in the colony. Since dosages for hamsters and swine, calculated on the basis of body weight, may not be applicable for mice, preliminary treatment trials were made to establish an effective regimen. After such treatment was shown not to have a deleterious effect on productivity, the entire colony was treated. The therapeutic regimen, management of the colony during treatment, and results obtained are described in this report.

## PRELIMINARY TREATMENT TRIALS

Naturally infected, seropositive mice, selected from discarded breeding females, were used in these studies. Their serologic status was determined by agglutination-lysis tests on serums collected by clipping the tail and expressing three to four drops of blood into a tube containing 0.5 ml saline. The resulting serum dilution (estimated to be 1:5) was tested for antibodies against *L. ballum*. All mice used had titers of 1:100 or greater. As ascertained in previous studies (Stoenner, 1957), kidneys of these mice invariably contained leptospires.

Groups of 20 such mice received varying quantities of chlortetracycline hydrochloride in their drinking water for 7 days (Table 2). To insure stability, solutions of antibiotic were adjusted to pH 2.3 with N hydrochloric acid solution. Eight days after termination of the course, serums for agglutination-lysis tests were obtained by cardiac puncture (0.5 ml blood

TABLE 1

*Distribution of antibody titers and incidence of infection among representative lots of discarded breeding females before and after chlortetracycline hydrochloride therapy*

| Date tested | Antibody titer* | | | | | Culture of kidneys |
|---|---|---|---|---|---|---|
| | Neg. | 1:10 | 1:100 | 1:500 | 1:5,000 | |
| 12-14-56 | 6 | 4 | 16 | 28 | 15 | 63/69† |
| Treated 6-21 to 7-15-57 | | | | | | |
| 8-23-57 | 12 | 4 | 3 | 5 | 0 | 9/24 |
| 8-29-57 | 30 | 3 | 3 | 2 | 0 | 9/38 |
| 9-19-57 | 31 | 7 | 2 | 0 | 0 | 9/40 |
| 10-16-57 | 35 | 4 | 2 | 0 | 0 | 9/41 |

* Agglutination-lysis test.

† Numerator = number of mice from which *L. ballum* was isolated; denominator = number tested.

TABLE 2

*Effect of 7 days of treatment with varying concentrations of chlortetracycline hydrochloride in the drinking water on the elimination of* L. ballum *from kidneys of infected mice*

| Lot | mg. chlortetracycline hydrochloride/liter | Results of treatment |
|---|---|---|
| A | 62.5 | 18/20* |
| B | 250 | 5/20 |
| C | 500 | 1/18 |
| D | 650 | 0/20 |
| E (controls) | none | 20/20 |

* Numerator = number of mice from which *L. ballum* was isolated; denominator = number tested.

mixed with 1.5 ml saline) and then kidneys of each mouse were examined for leptospires. A small portion (about 2 mm diameter) from the right kidney of each mouse was incubated in a tube of Verwoort's medium for a minimum of 21 days. Since bacterial contaminants may interfere with the isolation of leptospires, only mice whose cultures of kidney remained free of bacterial growth are included in the data. Only one mouse of the group that consumed water containing 500 mg antibiotic/liter (Group C) yielded *L. ballum* while none of Group D that drank water containing 650 mg/L yielded this organism (Table 2). Since the antibiotic had eliminated the organism from most of the mice in Group C and from all in Group D, the dosage the latter group received appeared adequate. On the basis of measured water consumption, each mouse had received 5 mg antibiotic daily.

Before the entire colony was treated, it was necessary to determine whether such a dosage had an adverse effect on productivity of the colony. Chlortetracycline hydrochloride (650 mg/L) was given for 9 days to 171 females mixed with 57 males in jars, each containing one male/three females. Production from this group was compared with that of an equal number of untreated controls housed under similar conditions. Surprisingly, females in the treated group conceived more readily than those in the control group. Fifty-three days after the experiment was begun, all treated females had become pregnant whereas only 64 per cent of control females had conceived. During this period, four females and three males in the control group died but all treated mice survived. Six per cent of litters from the control group and 3 per cent of litters from treated females died from various causes before regular weaning time. At 21 days of age litters of treated mice were obviously more thrifty than litters of control females. Furthermore, mice in litters of the treated group were more uniform in size.

### TREATMENT OF THE COLONY

A brief description of methods of husbandry used at this station is necessary to understand the management of the colony during treatment. About 10,000 breeding stock are housed in a separate building, 32 feet × 100. All facilities for caring for the colony, such as cleaning, washing, and sterilizing jars and cans, and storage of feed and bedding are available in this building. About 50 per cent of the mice are held in jars containing one male and three females. When obviously pregnant, females are placed in individual jars where parturition occurs. From 4,000 to 5,000 dams with young of varying ages are cared for in such jars. Litters that are not used for experimental purposes are weaned at 21 days of age. After the young are weaned, females are returned to a male for breeding.

A pelleted feed[1] is fed ad libitum to mice of all ages. After 15 April, 1957, stock kept in breeding jars were fed pellets containing 100 gm chlortetracycline hydrochloride/ton of feed in an attempt to control infantile diarrhea. This dosage of anti-

[1] Purina Laboratory Chow.

biotic did not affect the incidence of leptospirosis in the colony. On the bases of preliminary treatment trials and the known rate of food consumption of adult mice (5 gm/day/25-gm mouse), the feed had to contain approximately 9 times as much antibiotic to eliminate leptospires from the kidneys of infected mice. To insure eradication of *L. ballum*, all adult and immature mice, which would later be used for breeding purposes, were fed pelleted feed containing 1,000 gm chlortetracycline hydrochloride[2]/ton for 10 days.

Treatment of the colony began 21 June, 1957 and terminated 15 July, 1957. The entire colony could not be treated simultaneously because baby mice nursing treated mothers were not suitable for certain experiments involving antibiotic-sensitive agents.

Mice in breeding jars and females in individual jars whose young were less than 7 days old were treated first. Mothers whose young were older on the date medication of the colony began were not treated until their litters were weaned. As litters were removed, mothers were placed in stainless steel cans in groups of about 30 per can. These received antibiotic feed for 10 days before being returned to males for breeding. Twenty-one-day-old mice reserved for future breeding stock were likewise treated for 10 days immediately after weaning. Weanling mice selected for experimental purposes were not treated. On the seventh day of treatment of a group of mice, *i.e.*, adults in jars or groups of 30 in cans, mice were changed to clean, sterilized containers. At this time water bottles were also washed and sterilized with flowing steam.

Since all mice in the colony were not treated simultaneously, precautions were taken to insure medication of every mouse. Traps were set and DDT was spread on likely runways to destroy mice that escaped from containers.

Periodically, after treatment, lots of discarded breeding females were examined for leptospires by the methods previously described. *L. ballum* could not be recovered from such treated mice (Table 1). Also of interest is the apparent decrease in antibody titer in remaining serologically positive mice. Although one cannot be certain that *L. ballum* was eradicated from the colony without testing every mouse, failure to recover the organism from lots examined during 3 months post-treatment suggests that the disease has been eliminated.

[2] Aurofac 10.

## DISCUSSION

The percentage of breeding colonies of mice that are infected with *L. ballum* is not known but it is probably greater than is generally recognized. Since this leptospira does not cause obvious clinical disease in mice, the presence of the organism in a colony usually would not be suspected. Wolff *et al.*, (1949) recovered *L. ballum* from two of six colonies studied and in this country at least two colonies of white mice were found to be infected (Yager, *et al.*, 1953).

The means by which *L. ballum* was introduced into the colony at this station remains unidentified. This leptospira is frequently found in the common house mouse, *Mus musculus* (Yager, *et al.*, 1953). Hence, mice which occasionally infested the building in which the colony was housed prior to 1950, might have introduced the organism. Once present in the colony, contact among mice at breeding time perpetuated the disease. In fact, since infected mice remain carriers for life and since some direct contact among mice must occur in a colony, the disease would persist indefinitely.

Recognition of leptospirosis (*ballum*) among laboratory personnel who handled mice prompted efforts to eliminate this leptospira from the colony. Health of the colony was a secondary consideration. Although *L. ballum* does not cause obvious illness in mice, the extensive renal lesions observed in a large percentage of infected mice must affect their health. After leptospires were eliminated from the colony, thriftiness, production, and general condition of breeding stock were improved. However, feed containing 100 gm chlortetracycline hydrochloride/ton is still given to mice while they are in breeding jars. Since this antibiotic is known to have an effect on nutrition, periodic treatment while mice are in breeding jars also may be responsible for the improvement in general health observed.

## SUMMARY

*L. ballum* apparently was eradicated from a large colony of Swiss albino mice by feeding pelleted feed containing 1,000 gm chlortetracycline hydrochloride/ton for 10 days. After 7 days of such therapy, mice were transferred to clean containers equipped with clean water

bottles, both of which had been sterilized by stream. Traps and DDT in runways were used to destroy mice that escaped while treatment was in progress and also to prevent reintroduction of *L. ballum* by the common house mouse.

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# THELAZIASIS IN MAN

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Eye worms, nematodes of the genus *Thelazia*, were first described by Bosc (1819) based on a brief, unpublished description by J. B. Rhodes who had removed specimens from an ox in France. Since then a number of species of eye worms have been described from other animals. Infection in man has been thought to be accidental. Perhaps the strongest evidence of this is the small number of reported cases. The present paper reviews previous reports of human thelaziasis and gives data on three new cases in the western United States.

## HISTORICAL

Twelve cases of thelaziasis in man have been reported in the literature, nine of them in Asia where all the worms which were identified proved to be *Thelazia callipaeda*. These are listed as follows: four worms removed from the eye of a coolie in Peiping, China (Stuckey, 1917); two worms from the eye of a farmer in Fukien, China (Trimble, 1917); *T. callipaeda* larvae removed from a papilloma of the lower eyelid of a Canadian missionary in Peiping, China (Howard, 1927); 21 worms removed from a boy about ten at Changsintien, Wanpinghsien, China (Hsü, 1933); six worms taken from a Chinese truck driver from Kweiyang, Kweichow, China (Liang, 1945); a case reported from Peiping, China, by Chang (1948); a single specimen taken in 1927 from a girl at Hwanghedo, Korea (Nakata, 1934); four worms removed from a 15-month-old girl at Yercaud (Salem District), India (Friedmann, 1948); a single specimen taken from an 11-month-old boy from northwestern Korea in 1945 (Sillman, 1953).

Three cases of human thelaziasis have been reported from North America and have involved a different species, *Thelazia californiensis*. Kofoid and Williams (1935) noted the removal of three worms from a 42-year-old physician who resided in Porterville, California. That an arthropod was suspected of being a possible vector is indicated by their statement that a "gnat or some other flying insect got into his right eye . . . while he was on a trip in the Sierra Nevada mountains." Burnett *et al.*, (1957) have now incriminated two members of the house-fly family, Muscidae, as carriers of the eye worm in the United States. Hosford, Stewart, and Sugarman (1942) reported the extraction of six worms from a 35-year-old policeman in San Francisco. This patient had also been in the Sierra Nevada mountains a short time prior to the examination of his eyes. The third case was reported by Friedmann (1951): a single specimen of *T. californiensis* was removed by the patient himself, an entomological worker, who had been in the hills near San Diego where it is quite possible that he became infected.

This parasite, described by Price in 1930, has been taken from a number of different mammals but seems to be more frequently found in deer and dogs (Parmelee *et al.*, 1956). To date, infections by *Thelazia* in mammals have been noted in only three western states: California, Nevada, and Oregon (Parmelee *et al.*, 1956).

The worms are whitish in color, threadlike, about an inch in length when mature, and glide with a serpentine movement across the surface of the eye. They seem to harbor under the eyelid and, in animals other than human beings, under the nictitating membrane. Symptoms of thelaziasis include excessive lacrimation, conjunctivitis, corneal scarification and opacity and, in some cases, blindness (Fig. 1).

## UNREPORTED CASES

In a previous paper (Parmelee *et al.*, 1956) the writers alluded to a case of human thelaziasis which had not been previously reported. The cooperation of Dr. John M. Klar, Gardena, California, has made it possible to present further details of the case. A male, desert recluse about 70 years of age, asked to have worms removed from the eyes of his 11-year-old shepherd dog. He then asked that worms be removed from his own eyes. Neither the man nor the dog showed signs of conjunctivitis, but both had an excessive amount of lacrimation. Neither appeared to be particularly disturbed by the presence of the

[1] This investigation was supported by a research grant, E-951(C), from the United States Public Health Service.

FIG. 1. Eye worms, *Thelazia californiensis*, shown gliding across the ulcerated eye of a dog.

worms. Within the past 5 years the old man has come three times for treatment of his dog. He stated that in the Twenty nine Palms area, where he had been prospecting, the "wild animals" were infected with eye worms.

Dr. Phillips Thygeson, University of California Medical Center, and Dr. Paul Martin, San Jose, have very kindly supplied the writers with details of another unreported case. A half dozen or so eye worms were removed from a patient who had been hunting deer in the Sierra Nevada mountains. The man had typical conjunctivitis; no keratitis was present, and the infection cleared shortly after the worms were removed. The patient recalled having washed his face in brackish water, and the thought was expressed that the water might have been contaminated by deer or other infected animals. However, the writers feel that it is more likely that an arthropod vector was involved.

Dr. Robert D. Bethel of Oroville, California, has kindly supplied the following information on a third case: "In November, 1951, I saw a 55-year-old woman resident of a Sierra Nevada community of about 3,000 foot elevation. She had a slightly red eye with a feeling of a foreign body in it for the past month. Examination showed a rather marked follicular hypertrophy of the upper and lower tarsal conjunctiva. On everting the upper lid a round worm was found in the upper culdesac. The specimen was sent to the Parasitology Laboratory at the University of California in San Francisco and identified by them as *Thelazia californiensis*."

Undoubtedly cases of human infections by *Thelazia* are more common than might be suspected from the paucity of reports in the literature. The writers would appreciate any information sent in by readers on cases of thelaziasis that may have been seen.

SUMMARY

Reports of 12 human infections with eye worm (*Thelazia* sp.) were found in the literature. Three of these involving *T. californiensis* were reported from North America. Data on three additional cases are here presented. Two of these infections were probably acquired in the Sierra Nevada mountain area and the other in the Mojave Desert.

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# BIONOMICS OF *SABETHES CHLOROPTERUS* HUMBOLDT, A VECTOR OF SYLVAN YELLOW FEVER IN MIDDLE AMERICA[1]

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## INTRODUCTION

Shannon *et al.*, (1938) first called attention to the sabethine mosquitoes as possible vectors of yellow fever when they isolated the virus by injecting into mice a mixed pool of 88 wild-caught specimens of *Sabethoides*, *Limatus*, *Wyeomyia* and *Trichoprosopon*. The staff of the Rockefeller Foundation in Brazil later did a considerable amount of largely unpublished work with sabethine mosquitoes and yellow fever virus, mostly with negative or unsatisfactory results (Strode, 1951). Galindo *et al.*, (1950, 1951) and Trapido and Galindo (1956, 1957) based on epidemiological evidence, focused attention on *Sabethes chloropterus* as a vector of sylvan yellow fever in Middle America. Galindo *et al.*, (1956) transmitted yellow fever from monkey to monkey by the bite of *S. chloropterus* and Rodaniche and Galindo (1957) and Rodaniche, Galindo and Johnson (1957) isolated the virus from wild-caught females of this species in Panama and Guatemala.

The interest of this Laboratory in *Sabethes chloropterus* dates back to the first investigations on yellow fever carried out in Panama during the year 1949. Since then much time and effort have been spent in attempting to establish a laboratory colony and in acquiring basic information on the biology of this species which could be applied to advantage in transmission experiments with yellow fever virus. The results of these investigations are presented in this publication as a preliminary note, which may form the basis for future work on this and other species of the tribe Sabethini.

## ESTABLISHMENT OF A LABORATORY COLONY

The colony was started from large numbers of adults reared from eggs obtained from females captured in the Cerro Azul area of Panama by the method described by Galindo *et al.*, (1950). Freshly emerged males and females were placed in 1-meter cubical screened cages. The lower half of one of the sides of each cage had a large opening covered with a cloth sleeve and a sliding panel, through which objects could be taken in and out. A glass pane on the upper half of this side of the cage permitted close observation of the colony. The remaining sides and top were completely lined with thick muslin cloth carefully machine-sewn and taped to the edges to prevent trapping of mosquitoes between the cloth and the screen. The muslin served the dual purpose of helping to maintain favorable humidity inside the cage and of allowing quick detection of such pests as cockroaches, spiders or mites, which might invade the colony. A thick layer of wet cotton placed on top and a large pan of water covered with cheese cloth inside the cage served to maintain adequate humidity for the colony. During the dry season the colony room was tightly sealed and the temperature and humidity were regulated by means of a large electrical humidifier. In this manner the colony was maintained at temperatures which fluctuated between 23°C and 30°C and relative humidities between 70 and 100 per cent.

Females were allowed to feed on blood by exposing to their bites a trussed rhesus monkey or a guinea pig every day, except Sundays and holidays, from hour 0900 to midday. Sugar solution was available to the mosquitoes in the form of a soaked cotton ball hung from the top of the cage and changed daily.

In order to simulate closely the natural breeding sites of the species (Galindo *et al.*, 1951), a well-ripened section of bamboo about 4 inches in diameter, half filled with water, was used as an oviposition receptacle. The top of the bamboo was closed with a lid, and a hole 1 inch in diameter was drilled through the side. Eggs were removed from the bamboo every other day by pouring the water into a small enamel pan.

As the larvae hatched they were transferred to large pans and reared in a yeast-infusion medium. Because of the cannibalistic habits of the larvae discussed below, only specimens of the same

[1] This investigation was supported in part by the Research and Development Division, Office of the Surgeon General, Department of the Army; under contract No. DA-49-007-MD-655, and by Grant No. E-1941 of the National Institutes of Health, Department of Health, Education and Welfare.

instar were kept in one pan. Pupae were removed daily and placed in small finger bowls under a lamp chimney for emergence. Adult specimens were transferred to the colony cage from 12 to 24 hours after emergence. The colony has thrived under these conditions and has been maintained in our laboratory for over 2 years.

BITING HABITS

As pointed out by Galindo *et al.*, (1950, 1951) *S. chloropterus* is strictly diurnal in habits. In long-term observations carried out in Panama by Trapido and Galindo (Galindo *et al.*, 1950; Trapido and Galindo, 1957), it has been demon-

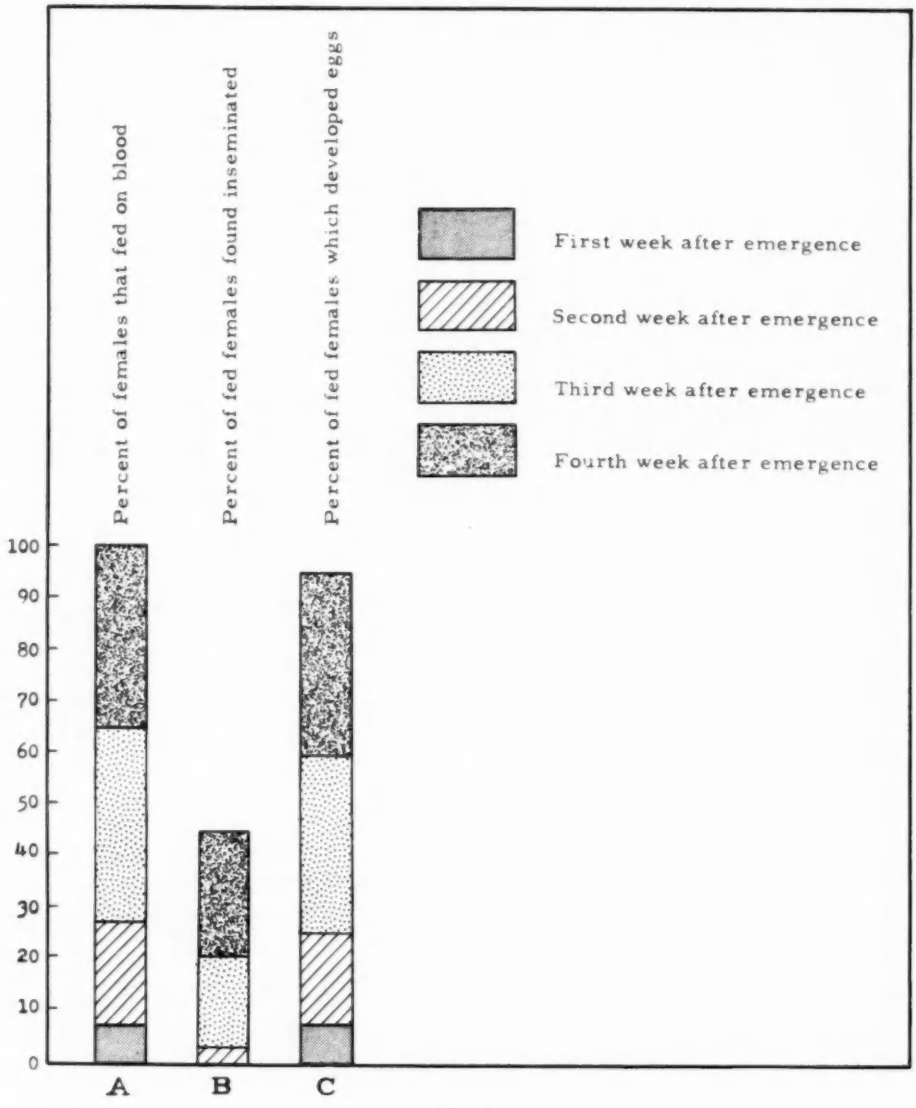


FIG. 1

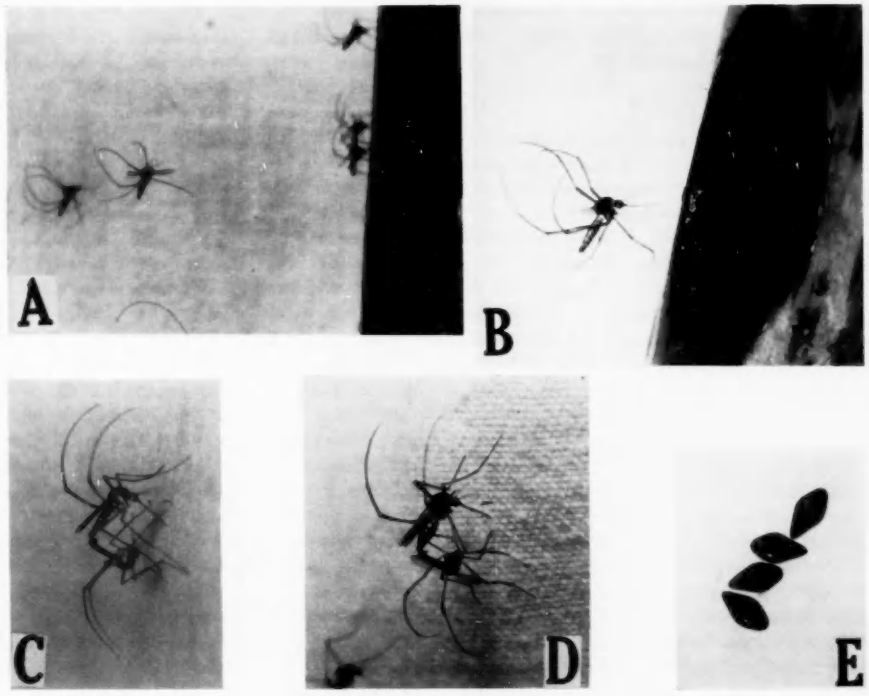


PLATE I

FIGS. A and B.—*S. chloropterus* females hovering in front of entrance hole, thrusting eggs into a bamboo pot half filled with water. FIGS. C and D.—*S. chloropterus* in the act of mating. FIG. E.—Eggs of *S. chloropterus*.

strated that this mosquito shows a daily biting cycle common to many neotropical diurnal forest mosquitoes, seeking a blood meal in greatest numbers during the early afternoon hours (1400–1500). This, in turn, corresponds to the midday rest period of most of the arboreal forest mammals which serve as hosts for this species.

In the laboratory, both males and females become engorged on sugar solution from 6 to 24 hours after emergence. No female has been observed feeding on blood before the fifth day after emergence. The peak of biting activity does not occur until the second or third week and it may be as long as 30 days before most females of a particular brood take a blood meal. Figure 1, column A, shows the results of an experiment in which 40 females emerging on June 3, were permitted to feed on a rhesus monkey every day from hour 0900 to 1200. These females were kept in a regular cage together with some 50 males and were treated in the same manner as the stock colony. On the fifth day after emergence the first female became engorged with blood, and by the end of the first week only 7.5 per cent had taken blood. At the end of the second week 27.5 per cent of the females had engorged, while at the end of the third week 65 per cent had bitten the monkey. This delay in taking the first blood meal had caused considerable trouble in transmission experiments on yellow fever with this species since freshly emerged females, which are usually employed in this type of work, invariably refuse to bite. Our practice now is to use for these experiments females that have previously fed on blood or those that show willingness to attack an animal introduced in the cage. This practice of using older females for transmission is feasible in this particular species since, as will be seen later, the mean life span of *S. chloropterus* is considerably longer than the normal extrinsic incubation

period of yellow fever. Females of *S. chloropterus* prefer to bite high on the body, having a special predilection for the nose and lips.

### MATING

*S. chloropterus* is a stenogamic species; that is to say, it is capable of mating in a small, confined space. Coupling of the sexes occurs while resting on a surface and it is preceded by peculiar epigamic activity on the part of the males. (See Plate I.) A male approaches a female from one side and with the mid-tarsus lightly taps the opposite hind leg of the female. It then hovers to the other side and goes through the same motion. This is repeated several times until the male suddenly pulls forward the mid-tarsus closest to the female, twists its body upside down and with a sliding motion attempts to drop directly under the female by seizing the surface of the wall with the fore-legs. The males usually overshoot their mark, in which case they may come back to start the epigamic motions all over again, fly to another female, or come to rest on the wall. If a male manages to get a hold on the wall, it immediately bends its abdomen upward and forward until it comes in contact with the tip of the female's abdomen and copulation begins. In 35 instances in which the time was measured, mating lasted from 14 seconds to 34 minutes, with an average of 6 minutes. During copulation the female remains motionless while the mid-tarsi of the male quiver rapidly, lightly tapping the tips of the female's antennae.

Copulation in this sabethine mosquito has not been observed before the sixth day after emergence and the peak of mating activity is not usually reached before the second or third week of the female's life. Columns B and C of Figure 1 show the results of an experiment in which 40 females kept in a colony together with 50 males were given the opportunity to feed daily on a rhesus monkey. Those females which engorged on blood were removed from the cage, held in separate vials for 6 days and then dissected to determine the rate of insemination and the number of specimens with fully developed ovaries. As can be observed, insemination does not necessarily occur before the first blood meal, and does not appear to be essential for ovarian development, since only 45 per cent of the females were found to be inseminated, while 95 per cent of the specimens showed full ovarian development.

### OVIPOSITION

The author (Galindo, 1957) has recently described the laboratory behavior of *S. chloropterus* while ovipositing in a bamboo internode with closed top and a 1-inch hole on the side, in the following manner: The female when ready to oviposit, approaches the bamboo in the characteristic slow flight peculiar to the genus and usualy flies around it two or three times probing here and there until the entrance hole is found. (See Plate I.) Once this is accomplished, the female hovers outside and in front of the opening at a distance of from a few mm to as much as 5 cm, with the fore and hind tarsi almost locked together above the thorax and the mid-legs extended downward and outward. After hovering for a variable length of time, and while still in flight, the mosquito suddenly jerks the head and thorax back and thrusts the abdomen forward, forcibly ejecting at the same time one or two eggs which shoot through the entrance hole and into the water in the cavity. Almost in the same movement the female darts back rapidly a few cm and then resumes normal flight. The entire process takes place with incredible speed and is completed in but fractions of a second. A female which has just laid may come back immediately

TABLE 1

*Rate of oviposition in* S. chloropterus *during the different hours of the day*

| Hour | 0600 to 0700 | 0700 to 0800 | 0800 to 0900 | 0900 to 1000 | 1000 to 1100 | 1100 to 1200 | 1200 to 1300 | 1300 to 1400 | 1400 to 1500 | 1500 to 1600 | 1600 to 1700 | 1700 to 1800 | 1800 to 0600 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aver. light intensity in ft. candles | 2.2 | 3.1 | 3.5 | 3.7 | 3.6 | 3.8 | 3.9 | 3.6 | 2.9 | 2.2 | 1.1 | 0.2 | — |
| No. of eggs | 191 | 312 | 397 | 576 | 449 | 630 | 855 | 670 | 990 | 603 | 584 | 280 | 0 |
| Per cent of total | 2.9 | 4.8 | 6.1 | 8.8 | 6.9 | 9.6 | 13.1 | 10.2 | 15.1 | 9.2 | 8.9 | 4.4 | 0 |

TABLE 2

*Rate of oviposition of* S. chloropterus *females during four periods of the day*

| Period of the day | Early morning 0600–0900 | Late morning 0900–1200 | Early afternoon 1200–1500 | Late afternoon 1500–1800 |
|---|---|---|---|---|
| No. of eggs | 900 | 1,655 | 2,515 | 1,467 |
| Per cent of total | 13.8 | 25.3 | 38.5 | 22.4 |

and go through the same motions for as many as eighteen consecutive times, or it may alight on the bamboo or some other surface nearby, only to resume egg-laying after resting for a few minutes. Tables 1 and 2 give the overall results of a series of experiments carried out during different seasons of the year, by which we determined the peak hours during which 6,537 eggs of *S. chloropterus* were laid.

As can be observed in Table 1, no eggs were laid in the total absence of light during hours 1800 to 0600. However, the position of the sun in the sky rather than light intensity seems to be the determining factor in inducing oviposition in this species. In our insectary cages light intensity is greatest in the morning, while the largest number of eggs were laid in the afternoon. In Table 2 the daylight hours are divided into four periods: early morning (0600–0900), late morning (0900–1200), early afternoon (1200–1500) and late afternoon (1500–1800). The greatest percentage of eggs (38.5%) were laid during early afternoon, while only slightly fewer eggs (22.4%) were deposited in late afternoon than in late morning (25.3%). The smallest number of eggs (13.8%) were laid during the early morning period. This peak of egg-laying activity during early afternoon also corresponds with the period of the day when the greatest number of females seek a blood meal.

*S. chloropterus* is a nonautogenous species; that is to say, it must have a blood meal (or its equivalent in certain proteins) before egg-laying takes place. As was pointed out when mating was discussed, copulation is not usually necessary to induce ovarian development, but a small percentage of virgin females fail to produce eggs even after fully engorging with blood several times. It must be borne in mind, however, that in some species of mosquitoes, such as *Aedes aegypti*, there is considerable variation of this physiological characteristic between local strains of the same species.

Table 3 and Figure 2 show the results of experiments made to determine egg production by day by *S. chloropterus* after a single blood meal. In these experiments, a stock colony was deprived of a blood meal until no eggs were laid for 10 consecutive days. A trussed monkey was then placed inside the cage and the fully engorged females were then collected and isolated individually in Barraud cages of 1 cubic foot, provided with bamboo sections about 1.5 inches in diameter, for oviposition. The water in the bamboos was examined daily for eggs during 15 days. As can be observed in Figure 2 oviposition began on the third day after the blood meal and went on through the thirteenth day. Most females oviposited during the fifth day. Table 3 indicates that, of 101 females which fully engorged with blood and which lived through the 15 days, 86 went on to lay a total of 3,323 eggs or 38.6 eggs

TABLE 3

*Egg production by day in* S. chloropterus *after a single blood meal*

| Exp. No. | No. of females | No. of females which laid | Days after blood meal | | | | | | | | | | | | | Total eggs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |
| 1 | 15 | 10 | 0 | 0 | 0 | 18 | 99 | 348 | 22 | 9 | 1 | 13 | 6 | 6 | 0 | 522 |
| 2 | 13 | 13 | 0 | 0 | 0 | 42 | 196 | 173 | 28 | 18 | 11 | 3 | 3 | 0 | 0 | 474 |
| 3 | 14 | 12 | 0 | 0 | 0 | 25 | 27 | 45 | 62 | 34 | 1 | 8 | 8 | 9 | 1 | 220 |
| 4 | 17 | 17 | 0 | 0 | 0 | 23 | 89 | 196 | 132 | 118 | 3 | 0 | 0 | 0 | 0 | 561 |
| 5 | 20 | 13 | 0 | 0 | 8 | 23 | 116 | 101 | 116 | 45 | 27 | 28 | 0 | 0 | 0 | 464 |
| 6 | 17 | 17 | 0 | 0 | 43 | 186 | 215 | 347 | 33 | 39 | 9 | 0 | 0 | 0 | 0 | 872 |
| 7 | 4 | 4 | 0 | 0 | 0 | 132 | 44 | 24 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 210 |
| Total | 100 | 86 | 0 | 0 | 51 | 449 | 786 | 1234 | 403 | 263 | 52 | 52 | 17 | 15 | 1 | 3323 |

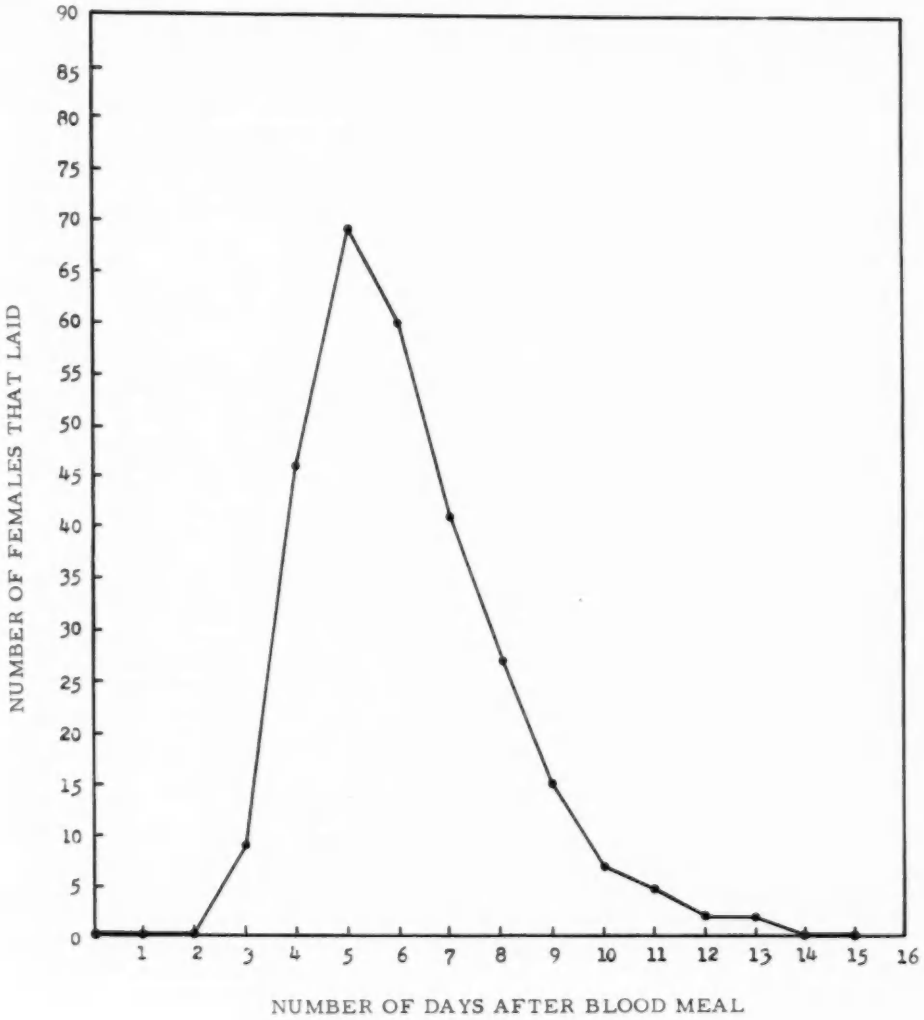


FIG. 2

per female. This table also shows that by far the the greatest number of eggs were laid during the sixth day, despite the fact that more females oviposited during the fifth day.

Experiments are still in progress to study the factors which determine the production of eggs, but it appears that, as in other species of mosquitoes, the size of the blood meal bears a direct relationship to the number of eggs produced. There are indications also that older females that have become blood-engorged several times tend to lay more eggs than those that have had but a single blood meal.

From the data presented in Table 3 it may be calculated that each female lays an average of 38.6 eggs every ovulation cycle and that one of these cycles is completed at colony-room temperatures every 11 days. If, as will be shown later, the mean life span after the initial blood meal of *S. chloropterus* females at these tempera-

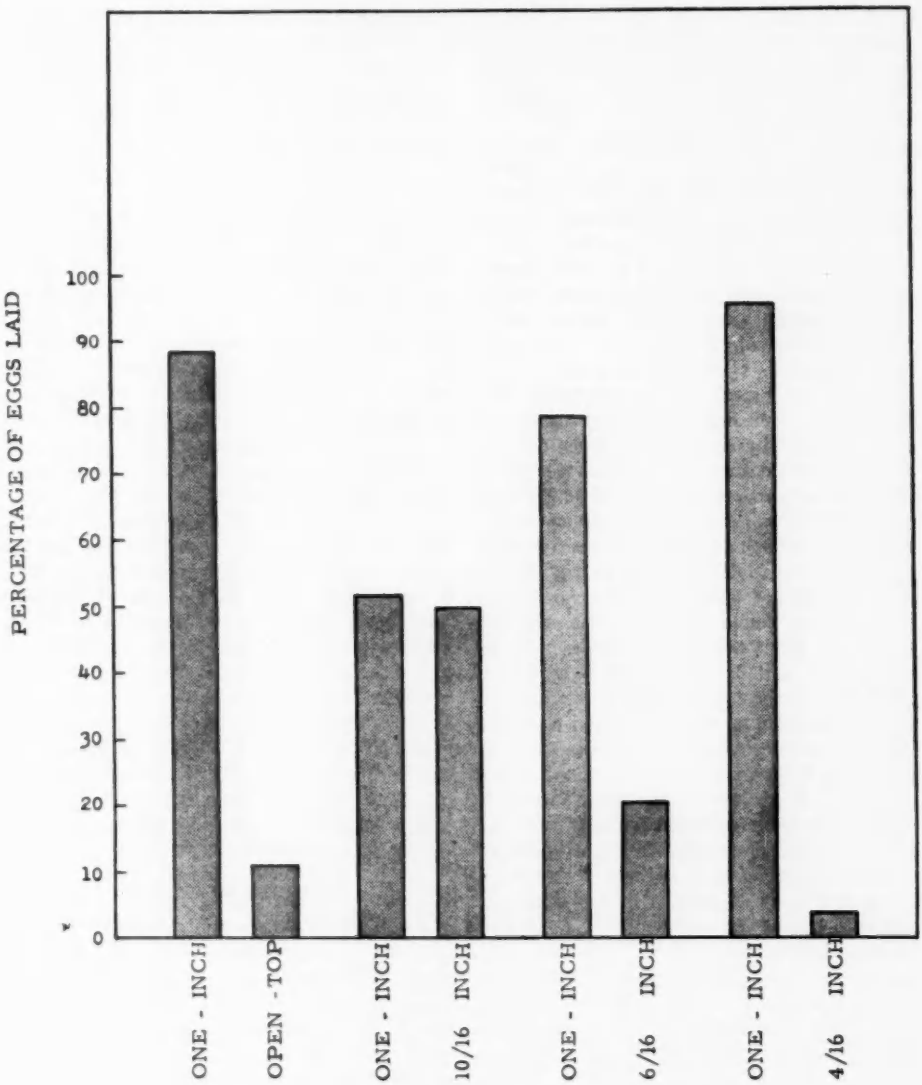


FIG. 3

tures is 38.9 days, then it would indicate that each female that survives through the first blood meal lays an average of 136.5 eggs during her life-time.

In order to check these figures, an experiment was made with three subcolonies totalling 382 females, in which we noted the total output of eggs from the initial blood meal until the last female died. It was found that these specimens laid 52,722 eggs or an average of 138.0 eggs per female, which checks very closely with the figure given above.

In Figure 3 are plotted the results of experiments set up to investigate the selection exercised

by *S. chloropterus* females when ovipositing in bamboos with different types of apertures. In the first of these experiments a bamboo with open top was placed inside a colony cage, together with a bamboo with closed top and a hole 1 inch in diameter drilled through the side. Eggs were removed daily from each bamboo and counted. The two bamboo pots were changed as to orientation and position every day, to avoid possible errors due to preference of the females for a particular spot in the cage. In the other experiments the same procedure was repeated but the open-top bamboo was eliminated and one with a 1-inch hole was paired with others having openings of 1⅛ inch, ⅝ inch and ⅜ inch.

In the first experiment, of 5,129 eggs laid, 4,579 (89.3%) were deposited in the bamboo with closed top and 1-inch hole, while only 550 (10.7%) were found in the bamboo pot with open top. In the second experiment, of a total of 13,063 eggs, 6,551 (50.1%) were laid in the bamboo with a 1-inch hole and 6,512 (49.9%) in the section with a hole 1⅛ inch in diameter. In the third experiment, a total of 6,832 eggs were laid, 5,392 (78.9%) being found in the bamboo with the 1-inch hole and 1,440 (21.1%) in the pot with the ⅝-inch hole. In the last experiment, of a total of 5,589 eggs, 5,367 (96.0%) were laid in the bamboo with a 1-inch hole and only 222 (4.0%) were deposited in the pot with the ⅜-inch hole. This preference for oviposition in a pot with a small lateral hole rather than in a section with open top is in agreement with the type of breeding place preferred by this species in nature, which consists of rot-holes with a large cavity and a small opening. Females do not appear to exercise any preference as between two bamboos having apertures of 1 inch and 1⅛ inch, respectively. As the smaller of these apertures is decreased to ⅝ inch and ⅜ inch there is a definite predilection for egg-laying in the bamboo with the 1-inch opening.

### THE EGG STAGE

The eggs of *S. chloropterus* are peculiarly rhomboid in shape, thus differing markedly from the known eggs of members of the tribe Culicini. (See Plate I.) It is interesting to note that eggs of this species are very similar to those of at least two species of typical *Sabethes*, namely, *S. cyaneus* and *S. tarsopus*, as well as to those of several species of *Wyeomyia*. However, they are strikingly different from the known eggs of *Trichoprosopon* (*digitatum*, *compressum*, *longipes*, *magnus*, etc), thus corroborating the close affinity that exists between *Wyeomyia* and *Sabethes*, through such an intermediate group as the subgenus *Davismyia*, and the wide divergence which evidently exists between these two genera and the more primitive sabethine genus *Trichoprosopon*.

The development of the embryo in *S. chloropterus* takes place somewhat more slowly than in most other mosquitoes, lasting from 3 to 4 days, but the eggs hatch soon after the larvae attain full development and are not capable of going into the prolonged diapause characteristic of some aedine species. In one experiment in which the time required for hatching 781 fertile eggs of *S. chloropterus* was recorded, the following results were obtained: 79.4 per cent of the eggs hatched on the third day after being laid, 18.4 per cent on the fourth, 0.9 per cent on the fifth, 1.2 per cent on the sixth and 0.1 per cent on the seventh day. The eggs of *S. chloropterus* are laid on the water surface and they may either float or sink to the bottom of the container, but there does not appear to be any difference in hatching response between the eggs on top or on the bottom. Eggs may be maintained for a few days on wet filter paper but they shrivel and die as soon as the paper becomes dry, while the larvae hatch in 6 or 7 days if kept moist. Eggs of this species can be successfully transported on journeys lasting no more than 6 days, by placing the eggs, soon after being laid, in Petri dishes containing wet cotton and lined with filter paper.

### THE LARVAL STAGE

Despite the fact that the larvae of *S. chloropterus* live in very dark tree-holes with a small opening to the outside, they do not show any signs of being negatively phototropic and become evenly distributed when reared in well-lighted pans. These larvae, as those of many other sabethine species, are facultatively predaceous and will feed voraciously on other mosquito larvae if given the opportunity. The author has observed this species preying on the following mosquito larvae in the laboratory: *Anopheles albimanus*, *Haemagogus equinus*, *H. lucifer*, *Culex quinquefasciatus* and *Wyeomyia scotinomus*.

The larvae of *S. chloropterus* are also definitely cannibalistic and will prey on smaller larvae of their own species. In spite of this marked cannibalistic habit, larvae can be reared successfully

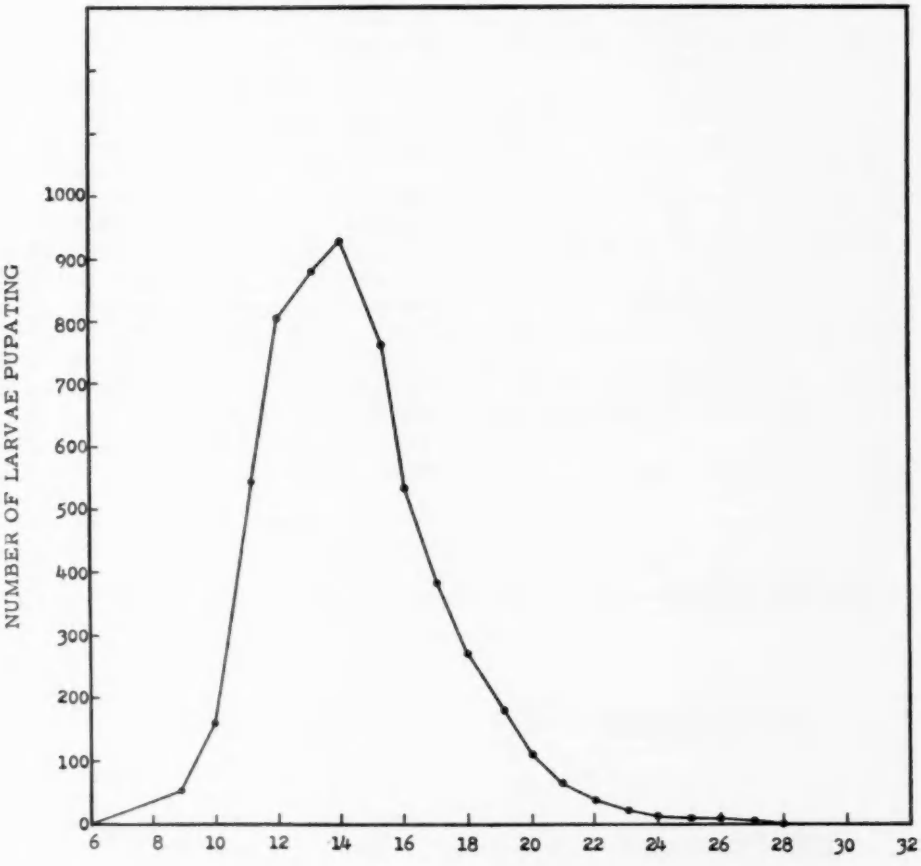


FIG. 4

in a yeast-infusion medium, provided that only specimens of the same instar are kept together. Even under these conditions a good number of larvae will attack and kill each other. This may be demonstrated by the fact that of over 32,000 larvae hatched and reared in our laboratory, in which the numbers of pupae were carefully counted, only 58.7 per cent reached the pupal stage. Galindo *et al.*, (1951) point out that predaceous sabethine larvae in general, and those of *S. chloropterus* in particular, do not swallow their prey but seize and puncture the cuticle with their hooked maxillae. Through this puncture and with the help of the mandibles, they gradually suck out the body contents of the victim and later drop the remains to the bottom of the container.

Figure 4 shows the overall results of a series of breeding experiments in which the rate of development of a total of 5,788 larvae was noted. These experiments were run at a mean bihourly temperature of the larval medium of 26°C, with a minimum of 24°C and a maximum of 30°C. The mean development period of these larvae from hatching to pupation was 14.8 days, with a maximum of 28 days and a minimum of 9 days. As would be expected, the rate of development depends a great deal on temperature and larval food. Larvae of this species have been observed to take as many as 49 days to pupate, with mean

TABLE 4

*Effect of crowding on larval development of* S. chloropterus

| Larvae per pan | Exps. | Total larvae | % pupae | Mean larval period (*days*) |
|---|---|---|---|---|
| 100–200 | 8 | 1174 | 57.8 | 14.7 |
| 200–400 | 9 | 2761 | 60.0 | 13.9 |
| 400–800 | 8 | 4510 | 62.3 | 14.3 |
| 800–1000 | 1 | 963 | 60.5 | 17.5 |

larval periods of 25.7 and 29.5 days, when fed exclusively on Pablum or on Pablum and live larvae of *Haemagogus equinus* and *Anopheles albimanus*. This would seem to indicate that yeast is one of the foods of preference for rearing this species in the laboratory. The addition of living mosquito larvae to the diet does not seem to accelerate development.

Table 4 gives the results of experiments made to determine the effect of crowding on the rate of pupation and on the length of the larval period. In these experiments we used round, white-enamel pans measuring approximately 10 inches in diameter at the water level. It may be noted that there appears to be no difference between the percentages of larvae reaching the pupal stage in any of the pans regardless of the number of larvae, ranging from 100 to 1,000. When more than 800 larvae were reared in a single pan there seemed to be a marked increase in the mean duration of the larval period.

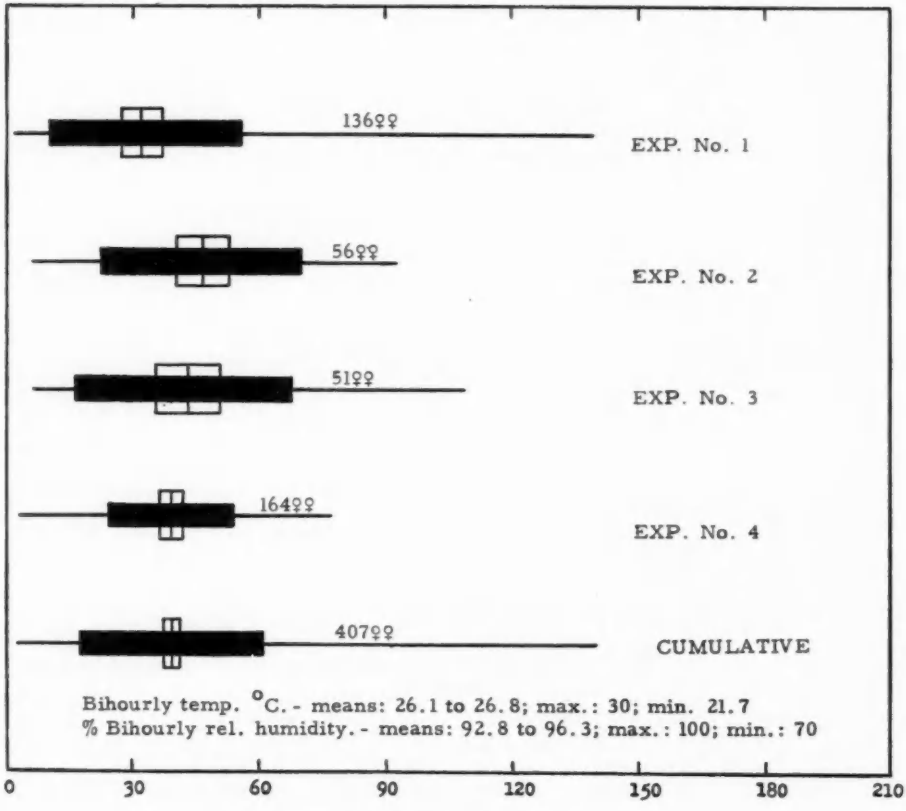


FIG. 5

### THE PUPAL STAGE

As in most other sabethines, the pupa of *S. chloropterus* develops very slowly. In one experiment run at a mean bihourly temperature of 26°C, in which the number of hours from pupation to emergence was measured for 1,607 pupae, the average length of the pupal stage was 150 hours with a minimum of 123 hours (one specimen) and a maximum of 160 hours (482 specimens).

### FEMALE LONGEVITY

Four experiments were made to determine the life span of *S. chloropterus* females under laboratory conditions in order to apply this knowledge to future transmission work with yellow fever virus. In these experiments, females were removed from a fresh stock colony immediately after engorging with blood for the first time, and transferred to colony cages. These mosquitoes were treated in the same manner as described earlier in this paper for the stock colony. If we consider that the life span in these experiments was measured from the time of the first blood meal until death, then it becomes obvious that the real longevity of these specimens is from 5 days to 4 weeks longer than shown, since it has already been pointed out above that *S. chloropterus* females do not begin to take blood until the fifth day after emergence and that it may be as long as 4 weeks before all the females of a particular brood take a blood meal.

In Figure 5 are plotted the results of the four experiments, as well as the cumulative data of all of them. The length of the thin center line in each of the columns represents the life span; the solid, broad, black stripe is one standard deviation from the mean; the hollow quadrangle represents twice the standard error and the cross bar is the mean duration of life in each case. As can be observed in this figure, while the maximum longevity showed much variation (from 78 days in Exp. No. 4 to 140 days in Exp. No. 1) the mean longevity (32.5 days in Exp. No. 1 to 46.8 days in Exp. No. 2) falls very nearly within the expected variation in random samples of a population, especially if it is considered that the four experiments were run under somewhat variable environmental conditions.

It is of interest to compare the cumulative mean longevity of the four experiments, which was 38.9 days with a maximum of 140 days, with that given by Jachowski (1954) for *Aedes polynesiensis* (the vector of filariasis in Samoa) which was 21.2 days with a maximum of 56 days.

TABLE 5

*Survival rate of* S. chloropterus *females after initial blood meal*

| Weeks after 1st blood meal | Per cent survival | | | | |
|---|---|---|---|---|---|
| | 1st exp. | 2nd exp. | 3rd exp. | 4th exp. | Cumulative data all exps. |
| 1 | 96.3 | 98.2 | 96.1 | 98.2 | 97.3 |
| 2 | 88.2 | 87.5 | 78.4 | 89.0 | 87.2 |
| 3 | 71.3 | 78.6 | 66.7 | 81.7 | 75.9 |
| 4 | 45.6 | 75.0 | 62.7 | 72.6 | 62.7 |
| 5 | 25.0 | 60.7 | 62.7 | 65.2 | 50.9 |
| 6 | 20.6 | 58.9 | 54.9 | 45.7 | 40.3 |
| 7 | 20.5 | 53.6 | 51.0 | 26.8 | 28.0 |
| 8 | 10.3 | 42.9 | 45.1 | 11.0 | 18.9 |
| 9 | 8.8 | 32.1 | 39.2 | 1.2 | 12.5 |
| 10 | 8.1 | 12.5 | 29.4 | 0.6 | 8.1 |
| 11 | 7.4 | 8.9 | 19.6 | 0.6 | 5.4 |
| 12 | 4.4 | 5.4 | 7.8 | 0 | 3.2 |
| 13 | 2.9 | 1.8 | 5.9 | — | 2.0 |
| 14 | 2.9 | 0 | 2.0 | — | 1.2 |
| 15 | 2.9 | — | 2.0 | — | 1.2 |
| 16 | 2.9 | — | 0 | — | 1.0 |
| 17 | 2.5 | — | — | — | 1.0 |
| 18 | 2.2 | — | — | — | 0.7 |
| 19 | 2.2 | — | — | — | 0.7 |
| 20 | 0 | — | — | — | 0 |

Table 5 gives the survival rate of *S. chloropterus* females in the four experiments mentioned above, as well as the cumulative data of all four experiments together. From the last column we may observe that 50 per cent of the females lived between 5 and 6 weeks after the initial blood meal, and that a mortality of 90 per cent did not occur until after the ninth week. It would be of interest to repeat these experiments under constant temperatures and to compare the longevity of this species with that of other known vectors of sylvan yellow fever, such as *Haemagogus spegazzinii*, *H. equinus* and *H. lucifer*.

### SUMMARY

A colony of *S. chloropterus* was established in 1-meter cubical cages. A trussed guinea pig or rhesus monkey served as the source of blood for the females, while a cotton ball soaked in sugar solution and hung from the top of the cage made available the necessary carbohydrates for both males and females. A well-ripened section of bamboo closed with a lid and with a 1-inch hole through its side was used as an oviposition re-

ceptacle. Larvae were reared in round enamel pans 10 inches in diameter in a yeast-infusion medium.

*S. chloropterus* was found to be strictly diurnal in habits. Females seek a blood meal in greatest numbers during the early afternoon hours (1400–1500). Both males and females become engorged on sugar solution from 6 to 24 hours after emergence. No female has been observed feeding on blood before the fifth day after emergence and the peak of biting activity is not reached until the second or third week. Females prefer to bite high on the body having a special predilection for the nose and lips.

*S. chloropterus* is a stenogamic species. Mating occurs while resting on a surface and is preceded by peculiar prenuptial activity on the part of the males. Copulation lasts from 4 seconds to 34 minutes, with an average time of 6 minutes. Mating has not been observed before the sixth day after emergence and the peak of mating activity is not reached before the second or third week of the female's life. Insemination does not necessarily occur before the first blood meal and does not appear to be essential for ovarian development.

Females oviposit by hovering in front of the hole in the bamboo section and forcibly ejecting one or two eggs through the hole and into the water in the bamboo. The position of the sun in the sky rather than light intensity seems to be the determining factor in inducing oviposition. The greatest percentage of eggs are laid in early afternoon (hours 1200–1500). The species was found to be nonautogenous, that is to say, it must have a blood meal before egg-laying takes place. Oviposition usually begins on the third day after a blood meal and goes on through the thirteenth day. The average number of eggs per female after a single blood meal is 38.6 with a minimum of 2 and a maximum of 103. Each female may lay an average of 138 eggs during her life time. Females exercise a definite preference for ovipositing in a bamboo with closed top and a 1-inch hole through the side rather than in a bamboo with open top.

The eggs of *S. chloropterus* are rhomboid in shape, the embryo develops in 3 to 4 days at room temperature and the eggs hatch soon after the larvae attain full development inside, being incapable of going into a prolonged diapause. The larvae are facultatively predaceous and cannibalistic but they do not frequently attack larvae of their own species, when in the same stage of development. They do not swallow the prey but suck the body contents through punctures in the cuticle.

The mean period spent by larvae from hatching to pupation at 26°C, is 14.8 days with a maximum of 28 days and a minimum of 9 days. The mean developmental period of pupae at this temperature is determined to be 150 hours with a maximum of 160 hours and a minimum of 123 hours.

The mean life span of females after the initial blood meal at mean temperatures of 26.1 to 26.8°C and a mean relative humidity of 92 to 97%, was found to be 38.9 days with a maximum of 140 days. It was also determined that 50 per cent of these females live between 5 and 6 weeks after taking their first blood meal and that 9 to 10 weeks elapsed before 90 per cent of these females died.

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# EPIDEMIOLOGIC STUDIES OF CONCURRENT "VIRGIN" EPIDEMICS OF JAPANESE B ENCEPHALITIS AND OF MUMPS ON GUAM, 1947–1948, WITH SUBSEQUENT OBSERVATIONS INCLUDING DENGUE, THROUGH 1957[1]

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WITH

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During the months of December, 1947 and of January through March, 1948 an outbreak of encephalitis occurred on Guam. This outbreak proved to be due in part to the virus of Japanese B encephalitis and in part to mumps virus. The studies undertaken at that time and which are reported here include a follow-up until 1957. It was found essential to include observations on dengue for reasons which will be readily apparent.

Guam is the largest of the Marianas group of islands and lies in the "tropical belt," about latitude 13° north. At the time of the epidemic it was an important military base with much heavy construction work in progress. The resident and native population numbered approximately 25,000 and the nonresident (military, civil service, contractors' employees, and dependents) numbered nearly 60,000.

Previous to the time of this outbreak no isolation of Japanese B encephalitis (JBE) virus had ever been made from, or any epidemic ever recognized as due to JBE virus, well within the tropics (Hammon, 1948). In fact, JBE was considered to be a disease of summer months occurring in areas of temperate or subtropical climate. Taiwan (Formosa), bisected by the Tropic of Cancer (latitude 23° north), with distinct seasonal variations in temperature appeared to be the most southerly extent of the recognized distribution of the clinical disease. However, antibodies to JBE or to an antigenically related virus had been found farther south in the sera of normal men or horses from the Malay Peninsula, Java, French Indo-China, the Philippines (Mitamura, 1943) and various parts of Africa. Subsequently, however, JBE virus has been isolated from the Federated States of Malaya (Paterson *et al.*, 1952), Singapore (Hale and Lee, 1954) and India (Work, 1956).

There is no evidence that, prior to 1947, any epidemic of encephalitis or even an endemic situation resembling JBE ever occurred on Guam. U. S. Navy medical records over a period of many years were examined and natives attested that no such disease occurred during the Japanese occupation of World War II. In 1899 a severe epidemic of a disease associated with flaccid paralysis, which was in all probability poliomyelitis, was reported by a U. S. Navy medical officer (Grunwell, 1900). It seems highly unlikely that this was JBE on the basis of the convalescent cases which were described.

During the first week of November, 1947 cases of mumps began to appear among the natives. This followed a probable introduction by American dependents. In certain of the northern villages mumps had not occurred for 6 or 8 years while, in the southern part, native estimates and the observed age groups in which the disease occurred fixed the time of the last epidemic at about 1930, a lapse of 17 years. Reporting of cases in the current outbreak was undoubtedly incomplete, but figures obtained from Captain C. K. Youngkin, MC USN, Director of the Department of Public Health, showed a rate as high as 192.5 per 1,000 in one village, with 1,647 cases occurring in a total population of 24,717, making a rate of 66.6 per 1,000. The incidence of the disease among American military and civilian personnel was of an expected order. Figures are available only for the military group, a rate of 2.5 per 1,000. Populations and rates for all groups for which data are available are presented in Table 1. The courses of the epidemics of the two

[1] These studies were conducted under the sponsorship of the Commission on Viral Infections, Armed Forces Epidemiological Board, and supported in part by the Offices of The Surgeons General, Department of the Army and Department of the Navy.

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TABLE 1

*Encephalitis and mumps cases and rates by population groups*

| Population group | Population* | Total encephalitis | | Cases of encephalitis from | | | | Parotitis only | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cases | Rate† | JBE | JBE-mumps‡ | Mumps | Unknown | Cases | Rate† |
| Resident population (Chiefly Guamanian)—Total | 24,717 | 46 | 1.86 | 13 | 14 | 14 | 5 | 1,647 | 66.6 |
| <15 years | 10,950 | 30 | 2.73 | 10 | 11 | 7 | 2 | — | — |
| 15 years and over | 13,767 | 16 | 1.16 | 3 | 3 | 7 | 3 | — | — |
| Nonresident population—Total | 57,972 | 8 | .13 | 3 | 1 | 3 | 1 | — | — |
| Military | 32,205 | 5 | .15 | 1 | 0 | 3 | 0 | 82 | 2.5 |
| Military dependents | 3,631 | 0 | .00 | 0 | 0 | 0 | 0 | — | — |
| Civil Service & Coast Guard employees & dependents | 4,408 | 1 | .22 | 0 | 1 | 0 | 1? | — | — |
| Contractor employees, dependents, etc | 17,728 | 2 | .11 | 2 | 0 | 0 | 0 | — | — |
| Total | 82,689 | 54 | .65 | 16 | 15 | 17 | 6 | — | — |

* As of January 1948.

† Per 1,000.

‡ JBE and/or mumps.

disease syndromes, mumps and encephalitis, are shown by weeks in Figure 1.

On December 6, a case of encephalitis was reported in a 22-month-old Guamanian child who had developed mumps 6 days previously. On Dec. 16th, encephalitis was noted in an American who also had mumps. Within a week several more cases occurred, some in persons without parotitis. All cases in natives were hospitalized at the Guam Memorial Hospital[5] under the care of U. S. Navy medical officers and all cases in military and American civilians were placed at the U. S. Navy Hospital.[6] The clinical and pathological aspects of these patients are reported separately by staff members of these hospitals, Edgren, Palladino, and Arnold (1958). A follow-up study of residual manifestations is reported by Pieper and Kurland (1958). No cases were seen at the Army Hospital.

Many of the illnesses observed during this early phase were very severe, presenting high temperature, disorientation, coma and convulsions. After 4 weeks occurrence of such cases a few terminated fatally. During the sixth week of the series of encephalitis illnesses an American marine died. Death occurred on the seventh day after the onset. His disease was not associated with mumps but he had no history of a previous attack of this disease. On January 19th central nervous system (CNS) tissue from several autopsies, including that of the American marine, together with serial serum specimens from the same and other patients were sent by plane to one of the authors (WMH) then in charge of the Neurotropic Virus Unit of the Hooper Foundation for Medical Research at the University of California in San Francisco. On February 2nd specimens of a similar nature from some of the same and other patients were sent to the U. S. Army's 406th Medical General Laboratory in Tokyo, Japan, then under the direction of another of the authors (WDT). In both laboratories viruses were quickly isolated and tentatively identified as JBE and serological tests performed (neutralization and complement fixation [CF]) confirming the diagnosis of JBE. Final and confirming identifications of the agents were reported at later dates.

As soon as the probable etiology was determined, field investigators at the invitation of the

[5] Capt. Harry C. Oard, MC USN, Commanding Officer.

[6] Capt. D. J. Wharton, MC USN, Commanding Officer.

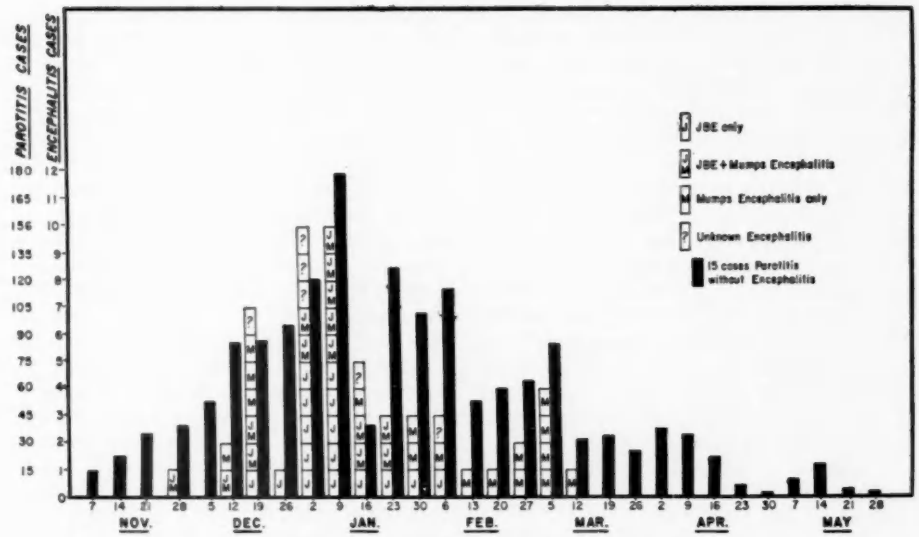


FIG. 1. Patterns of epidemics of encephalitis and mumps by weeks

Navy and the Army established a laboratory on Guam. This laboratory was activated on February 12th and continued to operate for about 2 months. Teams worked in essentially all inhabited parts of the island collecting blood from normal persons and from animals, collecting mosquitoes and other biting arthropods and obtaining epidemiological information. Diagnostic specimens, acute, convalescent and/or autopsy, were routinely obtained through the staffs of the two hospitals. Specimens of blood and autopsy materials, after samples were tested by CF, were divided on an alternate-person or animal basis and eventually taken in dry ice to the two base laboratories, Tokyo and San Francisco, for subsequent testing. In the meantime they were held in mechanically-refrigerated ice-cream-holding chests at about −10°C. Later sera were collected by members of the hospital staffs and sent under refrigeration to one or the other of the two laboratories.

The mosquitoes were all taken to the California laboratory where isolation attempts could be carried out without the potential hazard of naturally transmitted virus in the area. Results of the tests made on mosquitoes and other biting arthropods collected have been reported separately (Hammon *et al.*, 1949). Due possibly to temporary failure of a freezing unit while mosquitoes were held on Guam, but more likely to the postepidemic time when collected, no virus was isolated. Therefore, during November, December and January, one year later (1948, 1949) an even more intensive mosquito survey and collection was made, the results of which have been published by Reeves *et al.*, (1951). These arthropods were kept under dry-ice refrigeration at all times, yet no virus was isolated. However, no cases of encephalitis identified as of the JBE type occurred during this following year. On epidemiological grounds *Culex annulirostris marianae* Skuse was considered the most likely vector.

## LABORATORY METHODS

*Serological tests.* Two types of serological tests were performed on all of the diagnostic specimens and on many of the normal sera from man and animals when the quantity was adequate.

Neutralization tests were carried out by the method of serial dilutions of virus and undiluted serum. The sera were not inactivated. Serum and virus mixtures were incubated 2 hours at 37°C, then inoculated intracerebrally (i.c.) into mice 3 to 4 weeks of age. The method employed in both laboratories was essentially that described by Hammon (1956). All sera from the same patient or normal person were tested simultaneously

except in a few instances. In such instances or in others where results were difficult to interpret, a repeat test was made in the United States, where all specimens were finally assembled in one laboratory for any retesting needed.[7] The Tokyo laboratory used the Nakayama strain of JBE virus while the laboratory in the United States used the Okinawa strain. The only difference appeared to be that the latter strain gave a slightly higher neutralization index (NI) with some sera. Frequently, however, aliquots of the same specimen were tested in both laboratories and, in general, the results obtained were remarkably uniform. Neutralization indices were calculated for each serum. A NI of 50 or greater was considered positive, of 25 to 50 as equivocal and less than 25 as negative. Only rises in titer of tenfold or greater were considered diagnostic.

In addition to tests made with JBE virus, many neutralization tests were run with St. Louis encephalitis (SLE) virus, West Nile (WN) virus and California encephalitis (CE) virus strain BFS 283 (Hammon *et al.*, 1952).

Some sera collected in subsequent years were tested for neutralizing antibody with Murray Valley encephalitis (MVE) virus since by then the serological relationship between MVE and JBE had been recognized (French, 1952; Pond *et al.*, 1955) and the virus was available for tests. A number of these later sera were also tested for antibodies with Ntaya, Ilheus, Uganda S, Zika and Semliki Forest viruses.

A much larger number of sera were tested during the follow-up period with dengue viruses, types 1 and 2. For the neutralization test suckling mice were inoculated by the i.c. route (Meiklejohn *et al.*, 1952). Since sera were kept frozen no "accessory factor" from fresh serum was added (Sabin, 1950).

Complement fixation was performed with a slight modification of the method described by Casals (1947) but using benzene-extracted antigens made as described by Espana *et al.*, (1948). Antigens used in San Francisco were prepared by Espana as were part of those used on Guam and in Tokyo. In later work in Pittsburgh the same type of antigen was used but a plate method, modified from that of Fulton *et al.*, (1949), was adopted (Hammon, 1956) following comparison with the original tube method. No significant differences were noted in results obtained in Japan, on Guam and in the United States in so far as CF was concerned. The Nakayama strain of JBE virus was used in all this work. A battery of antigens was routinely employed in the CF tests. JBE antigen and a normal mouse-brain control were always included. Usually one or more strains of SLE, frequently WN, and less frequently Western equine encephalomyelitis virus (WEE) and/or CE virus were included. In the diagnostic tests on patient sera a mumps-virus antigen was also employed. This consisted of allantoic fluid from inoculated chick embryos and was made in the San Francisco laboratory. All tests with this antigen were performed there. A normal allantoic fluid was used as a control for this antigen.

In all CF tests 2-fold serum dilutions were employed beginning at 1:4. A 2-plus (++) or 3-plus (+++) reaction at 1:4 was interpreted as equivocal while a 4-plus (++++) reaction at 1:4 or a 2-plus reaction at 1:8 or anything above this was classified as positive. Both laboratories had had at least 2 years of experience with CF tests in this group of diseases and many hundreds of sera had been tested from Far Eastern and Pacific areas.

Hemagglutination inhibition (HAI) tests were performed only with dengue antigens, using the method of Sweet and Sabin (1954).

*Virus isolation and identification.* The methods used in both laboratories were again very similar. A 10 to 20 per cent emulsion of brain tissue was centrifuged and the supernatant treated with penicillin, then inoculated intracerebrally and intraperitoneally into groups of mice 3 to 4 weeks of age and intraperitoneally into suckling mice. Animals showing suspicious signs were sacrificed, their brains removed and cultured and an emulsion inoculated intracerebrally into others 3 or 4 weeks of age. Usually, after the second passage, identification was attempted by each of two methods.

A quantitative neutralization test was performed with several standard immune sera of known potency, including JBE antiserum, and a CF antigen was quickly prepared by centrifuging a mouse-brain suspension for 30 to 60 minutes at 16,000 rpm in an International Centrifuge Multispeed attachment. This antigen was tested

[7] This was done in the laboratory of the Department of Epidemiology and Microbiology of the Graduate School of Public Health, University of Pittsburgh to which two of the authors (WMH and GES) had moved.

TABLE 2

*Laboratory findings on fatal cases*

| Pt. | Nat.* | Age | Days after onset | Type specimen | Neutralization index JBE | Complement-fixation titer† | | | | Virus isolation | Diagnostic group |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | JBE | SLE | WEE | Mumps | | |
| W. P. | A. | 19 | 6 | Serum | 50 | <4 | <4 | <4 | 8 | | JBE only |
| | | | 7 | Serum | 1600 | <4 | <4 | <4 | 4 | | |
| | | | 10 | Serum | 5000 | <4 | <4 | <4 | 4 | | |
| | | | 10 | Brain | | | | | | JBE | |
| J. A. | G. | 1 | 8 | Serum | 280 | <4 | <4 | <4 | <4 | | JBE only |
| | | | 8 | Brain | | | | | | JBE | |
| M. D. | G. | 4 | 2 | Serum | 16 | 16 | —‡ | <4 | <4 | | JBE and mumps |
| | | | 4 | Serum | 50 | 32 | 16 | <4 | 32 | | |
| | | | 4 | Brain | | | | | | JBE | |
| D. F. C. | A. | 37 | 5 | Serum | 75 | <4 | <4 | <4 | 8 | | JBE only |
| | | | 7 | Serum | — | <4 | <4 | — | 8 | | |
| | | | 7 | Brain | | | | | | JBE | |
| C. D. | G. | 4 | 4 | Serum | 580 | <4 | <4 | <4 | <4 | | Unknown |
| | | | 4 | Brain | | | | | | None | (died too soon) |

* Nationality: A = American, G = Guamanian.

† CF titers given as reciprocal of dilution giving a ++ reaction or greater.

‡ Not done.

by overnight CF against standard guinea pig antisera (Hammon and Espana, 1947). This latter method was the most rapid but was accepted as tentative identification only. Neutralization test results were available about 1 week later. However, final identification was accepted only after confirmation by a mouse vaccination-challenge test since this test alone does not give any significant crossing with SLE virus or certain others in the antigenically related group.

### RESULTS OF SEROLOGICAL TESTS IN PATIENTS

Using all information available and the best clinical and laboratory judgment possible, all encephalitis cases were classified into one of four categories (Table 1): (1) JBE only, 16 cases; (2) mumps only, 17 cases; (3) JBE and/or mumps, 15 cases; and (4) unknown, 6 cases.

Further substantiation of the accuracy of these diagnoses was subsequently obtained by testing sera obtained from 29 of these same patients 10 years later. These 1957 sera[8] were all tested quantitatively for JBE NI. Among seven with the diagnosis of mumps encephalitis, none had detectable JBE antibody. In 17 with JBE or JBE and/or mumps encephalitis, only one was without detectable JBE antibody. Titers ranged from 100 to 2,700. In the unknown group, four sera were obtained. Three of these had JBE antibody. Generalizing from the above, it would appear probable that most of those with uncertain diagnosis were cases with JBE infection.

The same classification shown in Table 1 was furnished to Edgren *et al.*, and has been used in their paper describing clinical aspects of the outbreak (1958) and to Pieper and Kurland who used it in their study of late residual clinical findings (1958).

Table 2 presents the more pertinent laboratory data on the fatal cases. JBE virus was isolated from four of the five which died; three from the JBE-only group, and one from an individual believed to have had both JBE and mumps. In all five fatal cases the NI had attained a level of 50 or more before death and in one patient (W. P.) in whom death did not occur until the tenth day, the NI had risen to 5,000. No fatalities occurred among those with mumps infection only.

Table 3 presents selected examples of antibody findings in nonfatal cases. It may be observed from these data that the antibody response may vary in JBE infections, both in degree and in time as measured against the onset of clinical illness. The NI on the first day after onset may

[8] Collected and shipped through the kindness of Dr. S. Pieper, U. S. Public Health Service.

TABLE 3

*Results of serological tests on selected patients who survived**

| Pt. | Nat. | Age (Yrs) | Interval after onset† | NI JBE | Complement-fixation titer | | | | Diagnostic group |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | JBE | SLE | WEE | Mumps | |
| R. D. | G. | 11 | 7 d | 100 | 64 | — | <4 | 32 | JBE only. (Parotitis occurred 6 weeks before onset of encephalitis) |
| | | | 24 d | 10000 | >128 | — | <4 | 64 | |
| | | | 38 d | — | >128 | — | <4 | 64 | |
| | | | 11 mo | 13000 | 64 | — | <4 | 16 | |
| | | | 10 yr | 700 | — | — | — | — | |
| A. Q. | G. | 11 | 11 d | 1 | AC | — | — | — | JBE only |
| | | | 31 d | 500 | 8 | — | <4 | <4 | |
| | | | 47 d | 6300 | 64 | 512 | <4 | <4 | |
| | | | 11 mo | 1000 | 16 | 128 | <4 | <4 | |
| J. S. | G. | 6 | 10 d | 1 | AC | — | — | — | JBE only. (Parotitis occurred 5.5 weeks before onset of encephalitis) |
| | | | 23 d | 32 | 32 | 128 | <4 | — | |
| | | | 40 d | 400 | 64 | 64 | — | — | |
| | | | 11 mo | 32 | AC | — | — | — | |
| | | | 10 yr | 100 | — | — | — | — | |
| V. Q. | G. | 1 | 1 d | 1500 | <4 | <4 | <4 | <4 | JBE only |
| | | | 10 d | 15000 | 8 | <4 | <4 | <4 | |
| | | | 24 d | 4000 | 16 | <4 | — | <4 | |
| | | | 10 yr | 2700 | — | — | — | — | |
| J. M. | G. | 2 | 6 d | 1 | 8 | <4 | <4 | <4 | JBE only |
| | | | 15 d | 750 | 128 | 16 | <4 | <4 | |
| | | | 10 mo | 23000 | 16 | — | <4 | — | |
| | | | 10 yr | 650 | — | — | — | — | |
| W. A. | G. | 4 | 6 d | 1 | 16 | 16 | <4 | 64 | Mumps only (onset of parotitis 5 days before onset of encephalitis) |
| | | | 18 d | 1 | 32 | 16 | <4 | 128 | |
| | | | 23 d | 1 | 32 | 16 | <4 | 64 | |
| | | | 33 d | 5 | <4 | — | <4 | 64 | |
| L. Q. | G. | 1 | 5 d | 1 | <4 | — | <4 | 128 | Mumps only (no parotitis) |
| | | | 22 d | 1 | <4 | — | <4 | 128 | |
| | | | 13 mo | 1 | <4 | — | <4 | 8 | |
| | | | 10 yr | 1 | — | — | — | — | |
| J. S. | G. | 12 | 9 d | 1 | <4 | <4 | — | 32 | Mumps only (no parotitis) |
| | | | 28 d | 1 | <4 | <4 | — | >128 | |
| | | | 34 d | 1 | <4 | <4 | <4 | >128 | |
| E. B. | G. | 2 | 1 d | 2500 | 16 | 4 | — | — | Mumps only (no parotitis but intimate contact history) |
| | | | 15 d | 1000 | 8 | <4 | <4 | 32 | |
| | | | 30 d | 3000 | 8 | <4 | <4 | 32 | |
| | | | 9 mo | 4000 | <4 | — | <4 | 16 | JBE immune from fairly recent infection |
| P. B. | G. | 11 | 7 d | 50 | <4 | — | — | 128 | JBE and mumps (no parotitis to date mumps infection) |
| | | | 24 d | 800 | 128 | — | <4 | 128 | |
| | | | 11 mo | 13000 | 8 | — | <4 | 32 | |

TABLE 3—*Concluded*

| Pt. | Nat. | Age (*Yrs*) | Interval after onset† | NI JBE | Complement-fixation titer | | | | Diagnostic group |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | JBE | SLE | WEE | Mumps | |
| D. I. | G. | 2 | 13 d | 100 | 8 | <4 | <4 | >128 | JBE and mumps (no parotitis to date mumps infection) |
| | | | 30 d | 500000 | 32 | — | <4 | >128 | |
| | | | 44 d | — | 32 | 8 | <4 | 64 | |
| | | | 11 mo | 200 | 16 | 8 | <4 | 16 | |
| J. S. | G. | 16 | 6 d | 5 | <4 | <4 | — | — | JBE and mumps (onset of parotitis about 1 week prior to onset of encephalitis) |
| | | | 20 d | 13000 | 128 | 512 | — | — | |
| B. L. | G. | 15 | 1 d | 60 | 8 | — | <4 | 64 | JBE and mumps (no parotitis to date mumps infection) |
| | | | 15 d | 2500 | >128 | 64 | <4 | 64 | |
| | | | 27 d | 7500 | >128 | 128 | <4 | 64 | |
| | | | 10 yr | 1700 | — | — | — | — | |
| J. C. | G. | 7 | 2 d | 130 | 16 | — | | 128 | JBE and mumps (no parotitis to date mumps infection) |
| | | | 10 d | 280 | 128 | — | | 128 | |
| | | | 27 d | — | 128 | 64 | | 64 | |
| | | | 43 d | 5000 | 64 | — | | — | |
| | | | 10 mo | 2500 | 128 | — | | 64 | |
| | | | 10 yr | 2700 | — | — | | — | |
| F. D. | G. | 4 | 5 d | 80 | <4 | <4 | <4 | >128 | JBE and mumps (recent onset of parotitis) |
| | | | 18 d | 1600 | 128 | 256 | <4 | >128 | |
| | | | 23 d | 630 | >128 | — | <4 | >128 | |
| | | | 39 d | 10000 | >128 | — | <4 | — | |
| | | | 11 mo | 4000 | 16 | <4 | — | 64 | |
| | | | 10 yr | 1600 | — | — | — | — | |

* See footnotes to tables 1 and 2.

† Interval after onset: d = day, mo = months, yr = years.

be 1,500 (V. Q.) or 60 (B. L.), while in others it is still 1 on the tenth or eleventh days after onset (J. S.—6 years and A. Q.). The NI may attain a height of 500,000 by the thirtieth day (D. I.) or never rise above 400 by even the fortieth day (J. S.—6 years). By 11 months after onset the titer may be much higher than within the first month or it may have fallen. Case W. A., with presumably mumps infection only, was placed in the table to illustrate a CF phenomenon which occurred several times. Elevated CF antibodies to JBE and SLE are present in W. A., with no significant neutralizing antibody. These CF antibodies were for JBE and SLE but not for WEE. This was not classed as having a JBE infection at or about the same time.

A number of cases gave NIs of 50 or above to WN virus and fewer with SLE virus. In every instance, in Guamanians, the titer to JBE was much higher.

As will be discussed in more detail later in connection with test results in normal persons, serological findings in this area of the Pacific must be interpreted with caution. There was a known past experience of this population with dengue, epidemic parotitis was occurring, and evidence was accumulated to suggest possible prior experience with yet another member of the B group of viruses (or a closely related etiologic agent).

However, in a group of clinical cases, occurring over a comparatively short period of time, and with JBE virus repeatedly isolated in two laboratories from fatal cases, rising NIs to JBE were interpreted to mean current infection. Repeated tests on a patient giving a more or less constant, elevated NI could mean previous infection, or

current infection, providing the first specimen was taken at such a time that the titer had already reached the plateau level.

Complement fixing antibodies to JBE virus do not have the same specificity as neutralizing antibodies. In man, they may overlap extensively with SLE in particular. Depending on the time when the specimen was drawn, the sensitivity of the antigen, and the previous experience of the individual with other group B viruses, the serum titer to the infecting virus may not exceed that to another antigen. Occasionally the heterologous titer may exceed the homologous. In contrast with results reported by Hodes *et al.*, (1945) and by Sabin (1947) in studies of sera from Okinawa with very low titered antigens, the results of both participating laboratories in this study, with sera from outbreaks in Japan, Korea and Okinawa, indicated that SLE antigen, with certain sera, gave titers as high as, or even higher than, those with JBE. Although dengue virus is not closely related antigenically to JBE, a human serum with a high CF titer to dengue may give a low titer to JBE (Sabin, 1949, 1950) and possibly higher to MVE (Doherty, 1957). Furthermore, dengue CF antibody may persist for several years following infection. Whether at such a time it will still cross react with JBE is unknown. Furthermore, CF antibody without neutralizing antibody, as shown by W. A., (Table 3) was found to occur a number of times, presumably without JBE infection, in studies of natives of Ponape, Samoa and other Pacific islands (Hammon, 1953). For these reasons a positive CF antibody to JBE virus was not interpreted to mean JBE virus infection unless accompanied by neutralizing antibodies. CF antibodies to JBE are of short duration. If they have significance at all, they indicate recent infection. Rising titers associated with neutralizing antibodies were interpreted as suggestive of current infection.

In respect to CF tests on mumps patients, it soon became apparent that it was not reasonable to require a rise in titer to mumps antigen to classify the case as due possibly to infection by mumps, providing the level of antibody was 1:32 or above in at least two fairly early specimens. This was because, in a number of cases of obvious mumps encephalitis (no JBE antibodies of either type), the antibody titers to mumps were at a high but equal level in each of the first two or three serum specimens. This high level in an early specimen can be assumed to be due to S (soluble) antigen present in small amounts in the type of antigen preparation employed. The high level in such a case was accepted as an indication of probable or likely current infection which could contribute to the clinical encephalitis, unless there was a history of parotitis more than 2 weeks before. In the absence of clinical evidence of mumps, constant low levels of 1:4 or 1:8 in older Guamanians or Americans were interpreted as indicative of immunity due to a mumps infection at some previous time. Serological testing of American troops indicated that a history of previous mumps infection had little reliability. Many serological "susceptibles" with no parotitis developed high antibody titers to mumps virus from inapparent infection.

The greatest difficulty in interpretation lay in the group with high or rising titers of antibody to both viruses. Many of these had to be interpreted as simultaneous infections or as successive infections in which one was so closely related in time to the other that the cause of the clinical manifestations had to be classified as JBE and/or mumps.

## DESCRIPTIVE EPIDEMIOLOGY

*Age distribution.* The 46 cases of encephalitis in the resident native population, by etiological groups, are presented in Table 4 according to age. The age distribution of those probably due at least in part to JBE virus (first two columns) had its highest incidence in the group under 5 years of age but largely spared those under 1 year. Cases and populations above and below 15 years are given in Table 1. The crude morbidity rate for those due at least in part to JBE was 1.1 per 1,000, and that of children below 15 years of age, 1.9.

The three cases attributed entirely or in part to JBE among nonresidents (Table 1), two in Americans and one in a Filipino, were all adults, ages 19 to 37. Populations for male adult nonresidents are shown in Table 1, *i.e.*, excluding dependents, since these may be presumed to have been largely women and children.

Mumps encephalitis (Table 4) appears to have followed quite closely the probable age distribution of mumps cases although, unfortunately, the exact ages of cases of reported parotitis were not available.

*Sex distribution.* Incidence by sex was essentially equal among Guamanians for mumps encephalitis alone while incidence of cases of

unknown etiology was slightly in excess in males. In those possibly involving JBE virus the cases in males exceeded those in the females, the ratio being nearly 2:1. All nonresident cases were male, which was more or less to be expected on the basis of the populations involved.

*Geographical distribution.* The geographical distribution of all resident Guamanian cases of encephalitis is shown in Table 5 and of all cases in residents and nonresidents due to JBE only or JBE and/or mumps in Figure 2. The island, for the purpose of this study, has been arbitrarily divided into two zones, North and South. Only three native villages with a total population of about 3,000 were located in the Northern zone, but a very large American and nonresident population was present. The Southern zone had by far the greatest native population, about 22,000 persons, but a much smaller proportion of the American and other nonresident groups. Rates per zone for JBE and JBE-mumps in the resident (chiefly native) groups were essentially as follows:

| | Cases | Population | Rates/1,000 |
|---|---|---|---|
| North | 2 | 2,921 | 0.7 |
| South | 25 | 22,242 | 1.1 |
| Total | 27 | 25,163 | 1.1 |

Cases with possible JBE etiology (laboratory evidence) are spotted on the map in Figure 2, resident cases by numbers in a circle and nonresident in squares. Mumps encephalitis cases (not shown) appear to have been slightly more evenly distributed on a population basis (Table 5).

The four nonresident cases with JBE involvement (Fig. 2) were located as follows: one North and three South. No JBE cases were identified from the Northern zone, either in nonresident or resident populations, except in the narrow neck of the island immediately adjacent to the dividing line as drawn. The four cases that occurred among nonresidents were closely related geographically to native cases. With the extent of movement over the island by both residents and nonresidents, it would not be surprising if a number of infected persons were found in the Northern zone even though transmission of virus did not occur in that area.

*Distribution in time, and association with geographic and climatologic data.* In Figure 1 it should be noted that encephalitis cases are shown by date of onset while mumps are noted by date of reporting (not available by onset). Reporting is

TABLE 4

*Age distribution of Guamanian civilian cases by serological and clinical groups*

| Age | Serological and clinical groups | | | | Totals |
|---|---|---|---|---|---|
| | JBE | JBE-mumps* | Mumps | Unknown | |
| 0–11 mo. | — | 2 | — | — | 2 |
| 1–4 | 5 | 4 | 4 | 2 | 15 |
| 5–9 | 3 | 3 | 1 | — | 7 |
| 10–14 | 2 | 2 | 2 | — | 6 |
| 15–19 | 2 | 3 | 5 | — | 10 |
| 20–29 | — | — | 1 | 1 | 2 |
| 30–39 | — | — | 1 | — | 1 |
| 40+ | 1 | — | — | 2 | 3 |
| Totals | 13 | 14 | 14 | 5 | 46 |

* JBE and/or mumps.

TABLE 5

*Geographical distribution of civilian Guamanian encephalitis cases by serological and clinical groups*

| Zone | Serological and clinical groups | | | | Totals |
|---|---|---|---|---|---|
| | JBE | JBE-mumps | Mumps | Unknown | |
| North | 0 | 2 | 2 | 0 | 4 |
| Dededo | | | 1 | | 1 |
| Tamuning | | 1 | | | 1 |
| Tumon | | 1 | | | 1 |
| Guam Mem. Hosp. | | | 1 | | 1 |
| South | 13 | 12 | 12 | 5 | 42 |
| Sinajana | | 1 | 3 | 1 | 5 |
| Mong Mong | | 4 | | | 4 |
| Toto | 1 | 2 | 1 | | 4 |
| Piti | 3 | | | | 3 |
| Agana Heights | 1 | | | | 1 |
| Chalon Pago | | | 2 | | 2 |
| Yona | | | 1 | | 1 |
| Asan | | 1 | 2 | | 3 |
| Merizo | 3 | 1 | 1 | 3 | 8 |
| Talofofo | 3 | 3 | | | 6 |
| Umatac | 1 | | | 1 | 2 |
| Agat | 1 | | | | 1 |
| Santa Rita | | | 2 | | 2 |
| Totals | 13 | 14 | 14 | 5 | 46 |

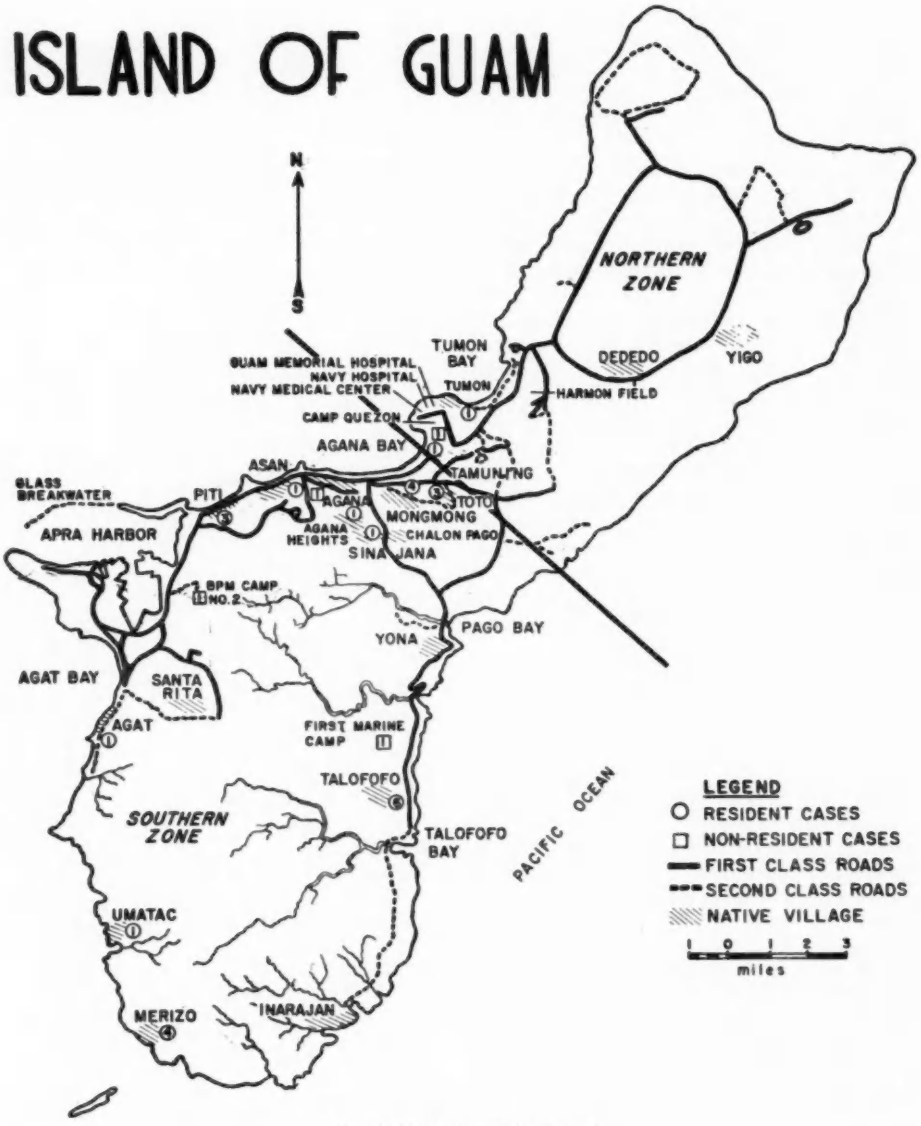


FIG. 2 *U. S. Army Photograph*

believed to have occurred on the average approximately 1 week after onset. In general these two curves (parotitis and all encephalitis) are almost identical. Insofar as the various subdivisions of the encephalitis categories are concerned, the pure mumps encephalitis tends to persist longer than the others, with a slight secondary peak in March, while those with JBE or JBE and/or mumps involvement, and the unknown, all followed a general pattern, reaching a peak about the week of January 9th and terminating by the week of February 6th.

The time sequence and the geographical distribution of all patients with probable JBE infection failed to show any radial spread or other systematic course. The first recognized case with a JBE component occurred at Mong Mong in the central neck of the island in the last of November while the next case occurred approximately 18 days later at Talofofo on the southeast coast. The epidemic ran its course in Talofofo with six cases in less than 3 weeks, during which time cases continued to occur in the central area involving Toto and Piti. While activity continued during the rest of the outbreak in Mong Mong and Toto and this general area, cases also occurred in Merizo and other southern areas. The first southern case other than in Talofofo was in Merizo on January 3rd, at about the midpoint of the epidemic. The four cases observed there were all within a 15-day period. On the other hand Mong Mong, in the central portion, had four cases spread over 2 months.

Associations between the occurrence of JBE and climatologic data were sought in an attempt to discover any unusual factors which might have been active in 1947–48. Since JBE elsewhere had been established as a mosquito-borne disease, rainfall and temperature as they might affect mosquito breeding and virus multiplication in the vector were of particular interest.

From various local sources good background information was available which may be summarized as follows. From December to June the rainfall is largely of the shower type. During the "winter," weak fronts of polar origin invade this area about four times monthly causing rainy weather for 1 or 2 days. The rainy season lasts regularly from about the 1st of July to the early part of November. Most of the "summer" precipitation is of the heavy-shower type with some squalls and thundershowers. Rainfall averages 90 inches a year with 59 inches falling during the period July through October. Throughout the remainder of the year rainfall averages 5 inches monthly with April the driest month, averaging 2.2 inches. There are 15 rainy days on the average in January and May, 12 in February and March, 9 in April, and 20 per month from July to December. Relative humidity is very high, averaging 84 per cent in January and February and increasing to 90 per cent in June.

The mean monthly temperature is relatively constant throughout the year with 79°F in January and February and 83° in June. The absolute maximum is 94° in May and June and the minimum 64° in February.

Figure 3 presents the rainfall by 5-day periods and the daily average temperature and relative humidity for a period slightly in excess of 1 year, including the period of the JBE encephalitis epidemic, December through February and the same months during the preceding year. For further comparison with the normal, Figure 4 shows the monthly mean and the maximum recorded precipitation based on records of many previous years and the actual monthly precipitation for periods of current interest.

Although nothing definite can be concluded from these data, in contrast to observations which would be desirable but unavailable on mosquito populations and biting activity during these periods, certain associations may have a relationship bearing on factors basic to the epidemic. Temperatures during December and January of 1947–48 averaged 1 to 2 degrees higher than during the preceding year. Relative humidity during December, January and February of 1947–48 averaged about 4 per cent higher. The rainy season in the "summer" of 1947 began more gradually and its peak was delayed until October, while November, usually with 7.5 inches of precipitation, had 12.5 inches. December still was above average and January, usually one of the driest months, had a rainfall of 9.3 inches. The rains finally stopped, making February an unusually dry month with 1.1 inches of rain and it may be assumed the mosquito population fell rapidly during this dry period. Thus, the total rainy season was prolonged from the usual period of 4 months to 7 months and despite the great rainfall during the early part of the usual dry season the temperature fell but little. These conditions may well have led to an unusually large population of the vector species and were compatible with a long enough period of build-up for mosquito infection rates to become very high.

### SEROLOGICAL FINDINGS IN THE GENERAL POPULATION

Elsewhere in the Far East it has been demonstrated repeatedly that many individuals may possess neutralizing and CF antibodies to JBE without a history of clinical disease. Various serological studies were conducted on Guam in an effort to measure the extent of infection during the

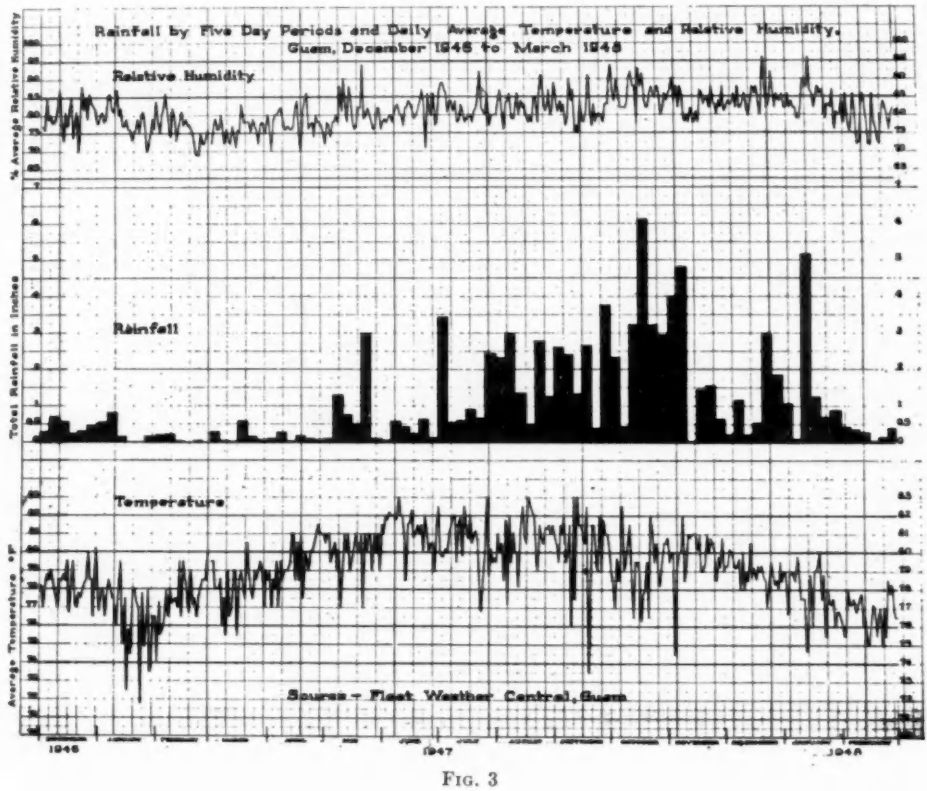


FIG. 3

current outbreak as well as to permit inferences as to whether this was a newly introduced disease in Guam or had been present for some period prior to 1948. Such an approach has many limitations. For example, the significance of a negative neutralization test in normal persons and animals cannot be determined with certainty on the basis of current knowledge. Antibodies have been shown to persist for 10 years (Table 3) and possibly may be life-long in persons suffering clinical disease. However, while duration of antibodies in those with a single inapparent infection is unknown, the experience of the authors in a very limited number of observations suggests that, in many persons, such antibodies may be quite temporary in nature. Therefore, a negative neutralization test in normal persons or animals is not interpreted to mean necessarily the absence of previous infection.

The converse of this is also true in the Western Pacific islands. Other members of the B group of viruses may produce low-level NIs against JBE. High CF levels have also been encountered which cannot at the present time be shown to be specific. One example of this CF response has been presented in the current evaluation of the clinical cases.

However, by following the changes of serological pattern in groups of natives over a relatively long period, and considering these in respect to their known overt disease experience, certain conclusions may be reached.

The only previous serological tests known to have been performed on Guamanians were made by Dr. Horace L. Hodes in 1945, then a member of the staff of the Navy Medical Research Unit No. 2. On inquiring by radiogram from Guam if tests had been made, he replied: "No extensive survey on Guam natives or animals made 1945, 4 horses and 4 natives had no antibody."

Several bleeding programs were carried out by our teams. The first of these involved normal native populations of all ages and was made in the latter part of February. This program continued through various geographical areas over approximately a month. A number of different military groups, hospital personnel and a group of student medical practitioners from other Pacific islands were included. Approximately 2 months after the sera were collected from certain of the native villages and one military installation, repeat specimens were obtained from the same persons to measure current infection rates. In late 1948 and early 1949 when the entomological team of Reeves and Rudnick return to Guam, other bleedings were made, including normal children as well as former patients. Again in 1953 and in 1957 blood sera were collected.

*Northern zone.* Table 6 presents the results of the serological tests by age groups from 117 persons from two villages of the Northern zone (Dededo and Yigo), from which no cases were recognized. There is no evidence of any antibody in the 0 to 4-year age group at any time, but 12 to 24 per cent of the older groups had neutralizing antibodies for JBE. Five persons had JBE CF antibodies and four others reacted to SLE only. Four of the five persons with elevated JBE CF antibodies also had elevated NIs to JBE (>2,500; 2,500; 2,500 and >1,000). Those with CF antibodies to SLE only had NIs to JBE below 50 with one exception, a level of 50 exactly.

The only nonresident populations bled from the Northern zone were personnel from Harmon Field, Guam Memorial Hospital, and the U. S. Naval Hospital and students at the School of Medical Practitioners[9] (natives of other islands). These, excluding the individuals of Micronesian origin, are grouped together in Table 7. Among the 66 nonresidents tested for NI, 6.1 per cent had significant titers. These few individuals had not been vaccinated against JBE while on duty elsewhere and their previous tours of duty had not included known endemic areas. They were not tested for SLE neutralizing antibody but were negative for WN. None of 116 tested for CF antibody was positive to JBE.

*Southern zone.* From the southern zone, insofar as nonresidents are concerned, only marines from the Yona area were sampled. The results of these tests on 52 persons are presented in Table 8. Two bleedings were made, one in February and one in April. Results of both tests are shown in the table. Only one person had antibodies of both types at the time of the first bleeding. Another developed neutralizing antibodies (NI of 100) by the time he was bled the second time, but his serum had no detectable CF antibodies. Four of the persons with equivocal levels of JBE neutralizing antibodies were found to have CF antibodies to SLE virus but not to JBE.

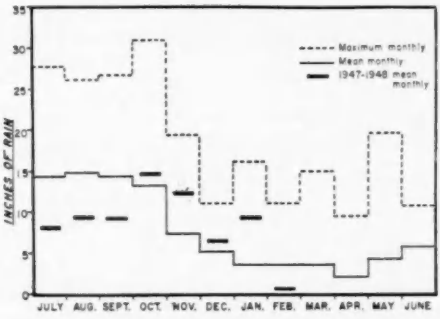


FIG. 4. Monthly mean and maximum precipitation for Guam and actual precipitation during period applicable to 1947–1948 encephalitis epidemic.

The most intensive native serological survey was conducted in the southern zone. The results by age groups from Merizo, Talofofo and Inarajan are combined in Table 9. Specimens taken 2 months apart were available from many. It may be observed that in each age group a much higher proportion of the population possessed JBE neutralizing antibody than in the Northern zone, ranging from 40 per cent in the youngest to 73 per cent in the 15 to 19-year age group. CF rates closely approached those for neutralizing antibodies, ranging from 36.4 per cent to 54.3 per cent. In the North, the CF rates ranged from 0 to 9.1 per cent.

Section B of Table 9 indicates that antibody response to infection continued to appear after late February, even though cases were no longer discovered. Several persons whose sera were free from detectable neutralizing antibodies in February had gained them by late April. However, many others who had CF antibodies earlier had lost them within 2 months.

*Natives of other Pacific islands.* Various serological findings which were difficult or impossible

[9] Cmdr. Sydney A. Britten, MC USN, Commanding Officer.

TABLE 6

*Northern zone, civilian Guamanians from villages of Dededo and Yigo bled between 2/6/48 and 4/2/48. Serological tests to JBE*

| Age | Neutralization | | | | | Complement fixation | | | | | Diff. % Pos.† Neut. & CF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. tested | Pos. | | Equiv. No. | Neg. No. | No. tested | Pos. | | Equiv.* No. | Neg. No. | |
| | | No. | % | | | | No. | % | | | |
| 0–4 | 29 | 0 | 0.0 | 1 | 28 | 29 | 0 | 0.0 | 0 | 29 | — |
| 5–9 | 24 | 3 | 12.5 | 4 | 17 | 22 | 2 | 9.1 | 2 | 20 | 3.4 |
| 10–14 | 25 | 6 | 24.0 | 0 | 19 | 18 | 1 | 5.6 | 0 | 17 | 18.4 |
| 15–19 | 18 | 3 | 16.7 | 0 | 15 | 18 | 1 | 5.6 | 0 | 17 | 11.1 |
| 20+ | 21 | 5 | 23.8 | 3 | 13 | 15 | 1 | 6.7 | 0 | 14 | 17.1 |
| Totals..... | 117 | 17 | 14.5 | 8 | 92 | 102 | 5 | 4.8 | 2 | 97 | 9.7 |

* Equiv. = Equivocal.

† Per cent positive by neutralization less % positive by CF.

TABLE 7

*Northern zone, nonresidents bled between 2/13/48 and 4/3/48. Serological tests to JBE*

| Neutralization | | | | | Complement fixation | | | | | Diff. % Pos.* Neut. & CF |
|---|---|---|---|---|---|---|---|---|---|---|
| No. tested | Pos. | | Equiv. No. | Neg. No. | No. tested | Pos. | | Equiv. No. | Neg. No. | |
| | No. | % | | | | No. | % | | | |
| 66 | 4 | 6.1 | 0 | 62 | 116 | 0 | 0.0 | 0 | 116 | 6.1 |

* Per cent positive by neutralization less % positive by CF.

TABLE 8

*Southern zone, marines of Yona Camp bled 2/18/48 and 4/13 48. Serological tests to JBE*

| Date | Neutralization | | | | | Complement fixation | | | | | Diff. % Pos.† Neut. & CF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. tested | Pos. | | Equiv. No. | Neg. No. | No. tested | Pos. | | Equiv. No. | Neg. No. | |
| | | No. | % | | | | No. | % | | | |
| 2/18/48 | 52 | 1 | 1.9 | 3 | 48 | 49 | 1 | 2.0 | 0 | 48 | −0.1 |
| 4/13/48 | 50 | 2 | 4.0 | 6 | 42 | 31 | 0 | 0.0 | 0 | 31 | 4.0 |
| 2 Spec.* | 52 | 2 | 3.9 | 8 | 42 | 49 | 1 | 2.0 | 0 | 48 | 1.9 |

* If either specimen was positive, it is classified here as positive.

† Per cent positive by neutralization less % positive by CF.

to interpret were seen in suspect encephalitis cases on Guam. Case W. A. (Table 3), for instance, shows a high CF to JBE and SLE but had no neutralizing antibodies for JBE. Similarly, the comparatively low levels of JBE neutralizing antibodies observed in some healthy individuals were puzzling.

For these reasons blood samples were drawn from healthy persons from nearby Saipan in late February. There had been no recognized cases of encephalitis on this island, yet 15 of 20 persons between 20 and 49 years of age had neutralizing antibodies to JBE. However, with one exception the NI was 500 or less. The exception was an 18-year-old male who showed also an equivocal NI against WN and low level CF antibodies to both JBE and SLE. Six of 15 (40 per cent) tested satisfactorily by CF were positive. Of these six positives, two had no JBE neutralizing antibody and one other had a NI of only 50. Several were

TABLE 9

*Southern zone, civilian Guamanians from villages of Merizo, Talofofo and Inarajan bled February and April, 1948. Serological tests to JBE*

| Age group | Neutralization | | | | | Complement fixation | | | | | Diff. % Pos.† Neut. & CF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. tested | Pos. | | Equiv. No. | Neg. No. | No. tested | Pos. | | Equiv. No. | Neg. No. | |
| | | No. | % | | | | No. | % | | | |
| *A. Combined results—2 specimens** | | | | | | | | | | | |
| 1–4 | 10 | 4 | 40.0 | 0 | 6 | 11 | 4 | 36.4 | 0 | 7 | 3.6 |
| 5–9 | 25 | 11 | 44.0 | 4 | 10 | 22 | 9 | 40.0 | 0 | 13 | 4.0 |
| 10–14 | 41 | 28 | 68.3 | 5 | 8 | 35 | 19 | 54.3 | 0 | 16 | 14.0 |
| 15–19 | 26 | 19 | 73.1 | 4 | 3 | 25 | 12 | 48.0 | 0 | 13 | 25.1 |
| 20+ | 45 | 30 | 66.7 | 4 | 11 | 45 | 19 | 42.2 | 0 | 26 | 24.5 |
| Totals | 147 | 92 | 62.6 | 17 | 38 | 138 | 63 | 45.7 | 0 | 75 | 16.9 |
| *B. Totals by time of collection* | | | | | | | | | | | |
| *Month* | | | | | | | | | | | |
| Feb. | 147 | 85 | 57.8 | 13 | 49 | 138 | 64 | 46.4 | 0 | 74 | 11.4 |
| April | 85 | 68 | 80.0 | 11 | 20 | 83 | 12 | 14.5 | 0 | 71 | 65.5 |

* If either specimen was positive, it is classified here as positive.

† Per cent positive by neutralization less % positive by CF.

TABLE 10

*Northern zone, School of Medical Practitioners bled 2/18/48. Serological tests to JBE*

| Normal resident of | Neutralization | | | | | Complement fixation | | | | | Diff. % Pos.* Neut. & CF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. tested | Pos. | | Equiv. No. | Neg. No. | No. tested | Pos. | | Equiv. No. | Neg. No. | |
| | | No. | % | | | | No. | % | | | |
| Samoa | 13 | 8 | 61.5 | 0 | 5 | 6 | 3 | 50.0 | 0 | 3 | 11.5 |
| Majuro | 4 | 1 | 25.0 | 1 | 2 | 3 | 0 | 0.0 | 0 | 3 | 25.0 |
| Rota | 2 | 1 | 50.0 | 0 | 1 | 0 | — | — | — | — | — |
| Ponape | 5 | 1 | 20.0 | 0 | 4 | 4 | 0 | 0.0 | 0 | 4 | 20.0 |
| Truk | 3 | 2 | 66.7 | 0 | 1 | 0 | — | — | — | — | — |
| Kwajalein | 7 | 1 | 14.3 | 1 | 5 | 5 | 0 | 0.0 | 0 | 5 | 14.3 |
| Palau | 2 | 0 | 0.0 | 0 | 2 | 1 | 0 | 0.0 | 0 | 1 | — |
| Yap | 4 | 0 | 0.0 | 0 | 4 | 3 | 0 | 0.0 | 0 | 3 | — |
| Saipan | 2 | 1 | 50.0 | 1 | 0 | 2 | 0 | 0.0 | 0 | 2 | 50.0 |
| Koror | 4 | 3 | 75.0 | 1 | 0 | 4 | 0 | 0.0 | 0 | 4 | 75.0 |
| Totals | 46 | — | — | — | — | 28 | — | — | — | — | — |

* Per cent positive by neutralization less % positive by CF.

positive by CF to SLE antigen and not to JBE and most of those with positive JBE CF had higher titers with SLE antigen.

At the School of Medical Practitioners in northern Guam no cases of encephalitis were seen. The students came from various Pacific islands and had been on Guam for periods varying from a few months to 3 years. Blood samples were also drawn from this group. The islands of origin and the results of serological tests are presented in Table 10.

Persons from several islands showed low level positive NI, none exceeding 1,000. This was not unexpected since similar findings had been obtained a few months earlier on natives from Ponape (Hammon, 1953). During the postwar

TABLE 11

*Guam animal population*

| Species | Prewar | 1948 |
|---|---|---|
| Carabao | 1,560 | 637 |
| Cattle | 5,845 | 2,855 |
| Goats | 1,353 | 366 |
| Swine | 14,089 | 4,218 |
| Horses | 126 | 56 |
| Chickens | 209,465 | 39,190 |
| Ducks | 1,027 | 363 |
| Other poultry | 932 | 265 |

years physicians of the U. S. Navy had observed sporadic outbreaks of an ill-defined febrile illness occurring among Ponape and Truk natives which, in some instances, showed evidence of neurological involvement and, in a few instances, left permanent neurological residua.

Among 71 sera drawn in September and October 1947, 64 per cent had CF antibodies to SLE (titers 1:8 to 1:32) and 29 per cent to JBE, but none to WEE or Russian spring-summer encephalitis viruses. However, none of these had neutralizing antibodies to JBE, SLE or WN viruses. There was no association of those with CF antibodies, in this instance, with history of a recent illness. The above tests were repeated and normal sera from the United States run simultaneously as controls and the results were confirmed. A smaller group of Ponape sera collected several months later from some of the same persons were found at this latter date to be free from CF antibodies but several by this time had NIs to JBE virus ranging from 25 to 50. These low NIs were confirmed by repeat tests.

In March, 1948 arrangements were made for blood samples to be collected from 50 "recovered" cases and 50 healthy persons. A single dose of formalinized chick-embryo JBE vaccine of the same type as used in Japan (Sabin and Tigertt, 1956) was then administered and a second blood sample secured 2 weeks later. It has been shown in Japan (Hammon, unpublished data; Sabin *et al.*, 1947) and in Okinawa (Tigertt and Hammon, 1950) that such a procedure would provoke a significant CF response in individuals recovered from a JBE infection. Both the control group and the "recovered" cases on Ponape contained individuals who showed initial CF levels against the JBE antigen varying from negative to positive at 1:64 dilution. SLE antibodies were also present but not necessarily in the same individuals as those with JBE antibodies. Vaccine administration evoked no significant change in the CF titers in either group, suggesting strongly that the agent or agents on Ponape, capable of eliciting both low-titered neutralizing and complement fixing JBE antibodies, was not JBE. It will be noted in Table 10 that, of the individuals at the School of Medical Practitioners, eight of 13 from Samoa possessed JBE neutralizing antibodies and three of six tested had CF antibodies. This was the only group which contained any persons with CF antibody. Persons from several islands possessed neutralizing antibodies. However, the NI of all positives was relatively low, none exceeding 1,000. Fifty per cent were tested also against WN virus, and NIs were either negative or lower than those to JBE virus.

As a result of these findings among Samoan students on Guam, one of the authors (WMH) conducted a separate serological study on natives in American Samoa in late August and September 1948 (Hammon, 1953) and found CF antibodies to SLE virus in titers from 1:8 to 1:128 in 50 per cent of a group of 16 hospitalized with a respiratory type of disease. Only 12.5 per cent reacted with JBE antigen and none with WN. However, only 19 per cent had detectable neutralizing antibody to SLE virus, all in low titer, and only 1 of 16 to JBE, and none to WN. In marked contrast to these were sera from 44 normal natives or patients with other medical or surgical illnesses. None had neutralizing antibodies to these viruses and of 24 tested by CF none was positive to these same antigens. However, 94 per cent of 68 of these sera from Samoa were positive when tested for neutralizing antibodies to dengue type 1 virus.

These data have been summarized here, not to attempt to define a disease entity, but to suggest that in various parts of Micronesia and Polynesia the serological patterns are such as to demand extreme care in the interpretation of any single test or group of tests as they pertain to JBE. On the assumption that the pre-epidemic pattern might be identical on Guam and these other islands, it is apparent that a positive CF test unaccompanied by a positive NI is of little etiologic significance, and that levels of JBE neutralizing antibodies which have been considered significant in Japan and Okinawa are open to question in Micronesia.

TABLE 12

*All areas, animals bled between February and April, 1948. Serological tests to JBE*

| Species | Neutralization | | | | | Complement fixation | | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. tested | Pos. | | Equiv. No. | Neg. No. | No. tested | Pos. | | Neg. No. |
| | | No. | % | | | | No. | % | |
| Horse | 4 | 4 | 100 | 0 | 0 | 4 | 2 | 50 | 2 |
| Pig | 10 | 10 | 100 | 0 | 0 | 5 | 1 | 20 | 4 |
| Carabao | 16 | 12 | 75 | 3 | 1 | 4 | 0 | 0 | 4 |
| Dog | 14 | 9 | 64 | 0 | 5 | 4 | 3 | 75 | 1 |
| Cow | 19 | 8 | 42 | 5 | 6 | 8 | 1 | 13 | 7 |
| Goat | 11 | 3 | 27 | 1 | 7 | 6 | 0 | 0 | 6 |
| Bat | 2 | 0 | 0 | 0 | 2 | — | — | — | — |
| Cat | 2 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 2 |
| Rat | 26 | 0 | 0 | 1 | 25 | — | — | — | — |
| Toad | 17 | 0 | 0 | 3 | 14 | — | — | — | — |
| Blackbird | 8 | 1 | 13 | 0 | 7 | — | — | — | — |
| Swift | 6 | 1 | 17 | 0 | 5 | — | — | — | — |
| Dove | 11 | 1 | 9 | 0 | 10 | — | — | — | — |
| Crow | 1 | 0 | 0 | 0 | 1 | — | — | — | — |
| Fairy tern | 3 | 0 | 0 | 0 | 3 | — | — | — | — |
| Rail | 6 | 0 | 0 | 0 | 6 | — | — | — | — |
| Chicken | 25 | 0 | 0 | 3 | 22 | — | — | — | — |
| Total | 181 | 49 | 27.1 | 16 | 116 | 33 | 7 | 21.2 | 26 |

*Infection in animals other than man.* At the time this outbreak occurred little was known regarding the source of mosquito infection in JBE but man, other mammals and birds were all suspected. An attempt was therefore made to determine as much as possible regarding the infection of these other animals. A reasonably accurate census of domestic animals was available over a period before and after World War II. A marked decrease occurred in the domestic animal population during the war and occupation, as illustrated by Table 11.

Table 12 presents the results of neutralization tests and a few CF tests on these animal sera. Animals were collected from many areas of the island but numbers of each species in any one place were too small to warrant treatment by areas.

### FOLLOW-UP STUDIES

During the late summer and fall of 1948 a large measles epidemic occurred with 5,000 cases and 13 deaths. The last previous measles cases had been during an epidemic in 1934 when 2,100 cases and 152 deaths were reported. Several cases of what appeared to be postmeasles encephalitis were observed in 1948 and autopsy material was submitted to the San Francisco laboratory from one patient. No mouse-pathogenic virus could be isolated from this specimen. Serological specimens also gave no indication of JBE virus etiology. Specimens of serum were submitted from three other cases of postmeasles encephalitis and tests showed no evidence of recent JBE infection.

Three cases of encephalitis were observed in 15- and 17-year-old Guamanian children without measles. Blood serum taken 4 days after hospital admission (onset dates not given) from each of these was submitted to the San Francisco laboratory. Two of the three had CF antibodies for SLE titering 1:64 and JBE titers of 1:16 and 1:32, with SLE NI of 25 and 50 but JBE NIs of only 1. All had mild meningoencephalitic illnesses not associated with coma. No conclusions could be drawn regarding etiology but it was felt that JBE virus was probably not the cause since, in general, neutralizing antibodies rise with, or earlier than, CF antibodies, and antibody to the related SLE virus is usually slower in developing than that to the homologous JBE.

Reeves and Rudnick (1951) made an extensive mosquito survey over the year's end, 1948–49. Properly frozen mosquitoes from this survey, tested in the San Francisco laboratory yielded no

TABLE 13

*Guamanian children. Serological tests, JBE, MVE, WN and Dengue 1 and 2*

| Age | Neutralizing antibody | | | | | | | | | | Hemagglutination antibody | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | JBE | | MVE | | WN | | Dengue 1 | | Dengue 2 | | Dengue 1 | | Dengue 2 | |
| | Pos. | Neg. | Pos. | Neg. | Pos. | Neg. | Pos.* | Neg. | Pos.* | Neg. | Pos.† | Neg. | Pos.† | Neg. |
| *Bled 1953* | | | | | | | | | | | | | | |
| 0–11 mo. | 0 | 7 | 0 | 7 | 5 | 1 | 3 | 2 | 5 | 0 | 5 | 0 | 5 | 0 |
| 1–5 yrs. | 0 | 58 | 0 | 58 | 0 | 52 | 0 | 49 | 0 | 48 | 0 | 42 | 0 | 43 |
| 6–10 yrs. | 1 | 27 | 1 | 27 | 2 | 27 | 4 | 22 | 7 | 19 | 6 | 20 | 6 | 20 |
| Totals..... | 1 | 92 | 1 | 92 | 7 | 80 | 7 | 73 | 12 | 67 | 11 | 62 | 11 | 63 |
| *Bled 1957* | | | | | | | | | | | | | | |
| 0–11 mo. | 1 | 5 | — | — | — | — | — | — | — | — | 1 | 5 | 1 | 5 |
| 1–4 yrs. | 0 | 12 | — | — | — | — | — | — | — | — | 0 | 12 | 0 | 12 |
| 5–9 yrs. | 0 | 26 | — | — | — | — | — | — | — | — | 0 | 26 | 0 | 26 |
| Totals..... | 1 | 43 | — | — | — | — | — | — | — | — | 1 | 43 | 1 | 43 |

* Pos. NI for dengue = 100 or greater.

† Pos. HAI for dengue = 1:40 or greater in 1953, 1:20 in 1957.

virus. These workers returned to San Francisco with sera from normal children 1 to 5 years of age from the three Southern zone villages of Merizo, Inarajan and Talofofo, and from children of similar ages from Dededo in the Northern zone and from children 3 to 15 years from the central portion just south of the line dividing the two zones (Fig. 2). These sera were tested in Pittsburgh for neutralizing antibodies against JBE and MVE viruses. Of 23 sera from the Southern zone only four (17%) were positive to JBE and none had a NI for MVE above 10. No positives to JBE occurred in children less than 3 years of age. None of five from the northern zone was positive to either virus, and from the central area one of five (a child 15 years of age) was positive to JBE only.

In late 1953 one of the authors (WMH) was able to visit Guam again. No cases suspected as JBE encephalitis had been occurring but 95 sera were collected at this time[10] principally from the northern and central areas. Children from birth to 10 years of age (born 1943 or subsequently) were included. All sera that were suitable were tested for JBE and MVE neutralizing antibody and most were tested for dengue types 1 and 2 neutralizing and HAI antibodies (Table 13). From 40 to 90 sera were also tested for neutralization with Ntaya, WN, Ilheus, Uganda S, Zika and Semliki Forest viruses. Only one serum of the 93 tested, that of a 9-year-old child (born 1944), had JBE neutralizing antibodies of a significant titer. He also had a lower NI to MVE, WN, Ntaya, and Ilheus viruses and was positive by neutralization and HAI to both dengue viruses. One infant 2 months of age had a NI to JBE of 34, and was positive also to WN and dengue. These antibodies were acquired undoubtedly from his mother. Several other infants less than 6 months of age did not have detectable antibodies to JBE, but most did to dengue and to WN. In no instance, however, were neutralizing antibodies detected to WN or any of the other agents employed with this group of sera unless antibodies to dengue viruses were also present. No antibodies to any viruses were present except in infants under 6 months of age and in children 9 years of age and over. The majority of those at these ages possessed such antibodies.

In March and April of 1957, 44 sera from children born during and subsequent to the termination of the epidemic in 1948 were received.[11] Only five were from the Northern zone, while 39 were from the Southern zone. Thus, the

[10] These sera were obtained through the kindness of Lt. Ronald L. Theile, MC USNR, U. S. Naval Hospital, Guam.

[11] Collected and shipped through the kindness of Dr. S. Pieper, U. S. Public Health Service.

majority of specimens came from the areas where infection rates had been highest during the epidemic of 1947. All sera were tested for neutralizing antibodies against 10 and 100 $LD_{50}$ of JBE virus and by HAI in dilutions of 1:10 and 1:20 against both types of dengue viruses (Table 13). All were without JBE antibody except that of one infant 2 months of age. This was from the Southern area. Serum of only one other infant under 6 months of age was included. At 5 months of age this infant's serum was negative. Twenty-one of the children were born in 1948, just after the epidemic. Only one serum possessed dengue HAI antibody, that of the same 2-month-old infant with JBE neutralizing antibody.

### DENGUE FEVER

Since dengue might have caused some difficulty in the interpretation of CF tests with JBE, and much more if HAI tests had been used, we felt it important to check serologically on the dengue situation. According to clinical reports this disease, previously present on the island, disappeared after a very large epidemic affecting American troops in 1944, followed in early 1945 by an intensive *Aedes aegypti* control program (Bohart and Ingram, 1945) which, with subsequent efforts, appears to have been successful in eradicating this vector from the island. One specimen only of *A. aegypti* was collected during our first study in 1948 (Reeves and Rudnick, 1951), none in 1948–49 (Reeves and Rudnick, 1951) and none subsequently by Hull (1952) or Hu (1953). *A. aegypti* had been considered the sole vector of dengue on Guam. *Aedes albopictus*, considered a vector together with *A. aegypti* on Saipan, was apparently not introduced into Guam until World War II. It still had a limited distribution as determined in our survey of early 1948 (Reeves and Rudnick, 1951), was much more widely distributed in the 1948–49 survey (Reeves and Rudnick, 1951) and was found essentially everywhere in 1951 (Hull, 1952). However, recognized clinical dengue did not reappear.

Since this evidence of the absence of dengue was quite convincing, little thought was given to dengue in the testing of sera when the 1947–48 encephalitis epidemic occurred, except that persons over 3 or 4 years of age were considered as possibly having been infected with dengue at some previous time. No tests were made, however, to check this until 1950 at which time very few sera remained. Sera from only three children under 4 years of age could be tested. None of these had neutralizing antibodies to dengue type 1, the only strain used in these tests. From 4 years of age and above only one person out of 15 tested did *not* possess detectable dengue neutralizing antibody. Meiklejohn, Stacy and Simpson (1954) when testing sera from adult Guamanians collected in 1946, reported five of 11 positive in the serum neutralization test against type 1 dengue. Their sera had not been frozen and considerable decrease in titer may have occurred.

Results of dengue tests on sera from 1953 and 1957 were presented in Table 13. These demonstrated clearly that dengue antibodies were present only in persons born before the *A. aegypti* eradication program, with the exception of infants under 6 months of age who had undoubtedly acquired the antibodies (both neutralizing and HAI) from their mothers, who probably were infected before 1945. In the sera taken in 1953 (Table 13), five infants possessed neutralizing antibody to dengue type 2, three of these to type 1 also, and all five had HAI antibodies to both dengue viruses. However, none of the five had JBE neutralizing antibody. This strongly suggests that maternally transmitted dengue antibodies do not cross-neutralize JBE virus and this same might well hold true for the original serum of the mother.

### DISCUSSION

*Clinical cases.* That JBE and mumps virus were both active simultaneously and producing disease cannot be doubted from the evidence obtained. It is of interest to consider whether the incidence of encephalitis was increased by a dual viral infection. If it were established that JBE virus had been endemic on the island for many years, the fact that this was the first recognized epidemic of encephalitis and was associated with the rare event of a "virgin" mumps epidemic might lend weight to the dual etiology hypothesis. However, since many cases of clinical encephalitis were shown to have occurred in the absence of a simultaneous mumps infection, the essential role of mumps virus in causing the syndrome must be discounted, at least in part. The same can be said for mumps encephalitis, since many mumps encephalitis patients were not infected with JBE virus. A dual hypothesis for etiology therefore, though interesting, cannot be established as essential, but may have contributed in those persons with simultaneous infections.

However, it is quite possible that JBE virus had not been present in inapparent, endemic form prior to the epidemic event observed. If this were established, no hypothesis of dual infection is needed to explain the sudden appearance of JBE encephalitis cases.

An additional point of interest in the clinical cases is the marked skew in the age distribution of Guamanian patients with probable JBE etiology. Eleven of 27 cases occurred in children under 5 years of age and only one case above 20 years (Table 4). If it is assumed that this is a "virgin" outbreak, is this possibly the true age-susceptibility pattern of JBE? Such an age distribution in the past has been interpreted as resulting from immunity possessed by the older age groups in an endemic area as a result of overt or inapparent experience with the virus. However, such an age distribution of clinical cases may be equally well explained on the hypothesis that the young are more likely to show frank clinical disease. Unfortunately, no proven virgin epidemic of JBE encephalitis is on record for comparison.

*Disappearance of JBE virus.* The serological findings from late 1948 through 1957, together with the absence of confirmed cases of JBE during these years, support only one conclusion, *i.e.*, JBE virus completely or practically disappeared from Guam immediately after the one recognized outbreak. This in itself is an unique observation insofar as our knowledge of the epidemiology of JBE or any of the other arthropod-borne encephalitis viruses is concerned, with the exception of Venezuelan equine encephalomyelitis in Trinidad (Downs, Anderson and Theiler, 1956). Its disappearance on Guam was certainly not due to any serious attempt to eradicate or control its vector. Little or nothing was done to increase mosquito control except in areas near American installations. Thus, the Southern zone, where infection was heavy, should not have been affected to any extent. Following the first investigation it was suggested to the administrative authorities that *C. annulirostris* was the probable vector, its breeding habits were described, and control measures suggested (Reeves and Rudnick, 1951). It was believed then that JBE virus had been present on the island for some time and that some unusual combination of circumstances had led to the epidemic. However, it was considered that epidemics might be expected to recur only at rare, unpredictable intervals, and that suitable mosquito control would make it much less likely to happen.

By now, *C. annulirostris* has become even more suspect as the vector. Hurlbut (1949) has demonstrated its ability to transmit JBE in the laboratory and Reeves *et al.*, (1954) have pointed it out on epidemiological grounds as the probable vector of MVE virus. Evidence that this mosquito has not been eradicated or controlled is found in the report by Hull (1952). In his survey of 1951, *C. annulirostris* larvae were collected from all parts of the island and in a great variety of habitats. It was reported prevalent in 1952 by Hu (1953) and, in 1957, Pieper wrote from Guam that an entomologist then present reported that it was currently found throughout the island.

From the data just presented it appears reasonable to conclude that natural conditions on Guam, though favorable for an explosive outbreak of JBE, were not suitable for the continued maintenance of the virus.

*Disappearance of dengue viruses.* The serological results of dengue tests reported indicate that almost all Guamanians living prior to 1944 had been infected with dengue and that persons born subsequent to 1945 or 1946 had escaped. If these interpretations are valid, no dengue infection of significance, and possibly none, had occurred on Guam beginning 2 or 3 years prior to the encephalitis epidemic of 1947–48. Since *A. aegypti* control became highly effective in 1945 and within a few years the mosquito had disappeared, it seems quite probable that the newly introduced *A. albopictus*, which is considered to be an important dengue vector on Saipan, did not play a significant role and that dengue virus itself disappeared from the island.

*Previous history of JBE virus on Guam.* As reviewed in historical data already presented there is no past evidence, clinical or serological, of the presence of JBE virus on Guam. It had been assumed by civil and military authorities not to be present and its isolation during the present outbreak came as a surprise. It is now our intent to review the total evidence to attempt to determine the situation prior to late 1947 when the epidemic began.

Much of the evidence to be considered is of a serological nature. Interpretation of serological patterns among normal persons is crucial for the determination of past infection rates. Unfortu-

nately, much remains to be learned in regard to serology of the group classified by Casals and Brown (1954) as the B group of arthropod-borne viruses, making it necessary to consider several possibilities. The most crucial group requiring interpretation in this respect is that with neutralizing antibodies to JBE but without CF antibodies. This group is usually considered to have had "previous" infection with JBE or an antigenically related agent. "Previous" implies not occurring during the same seasonal outbreak or possibly not as recent as 6 months.

Three possibilities will be considered in an attempt to interpret this serology in respect to what occurred in the population prior to the current epidemic: (1) all neutralizing antibodies at the level accepted as positive are the result of JBE virus infection but those not associated with CF antibody were acquired at least 6 months, probably years, before the blood was drawn; (2) all neutralizing antibodies are specific as assumed in (1), but were acquired during the current epidemic; thus CF antibody from inapparent infection is more labile than when acquired during clinical disease and in many persons had already disappeared in less than 6 months; (3) neutralizing antibodies not accompanied by CF antibody are due to repeated previous infections with dengue viruses or other group-B related agents. Any combination of these is possible, but one probably accounts for the majority of instances.

These three possibilities support one or the other of two hypotheses in respect to the past history of JBE virus on the island: (1) the first interpretation supports the hypothesis that JBE virus was previously present on the island, at least for a few years; (2) the second and third possibilities support the hypothesis that JBE virus had just infiltrated the island and that this was a "virgin" epidemic. Since the serological interpretations are not subject at present to experimental verification but all are reasonable possibilities, the evidence in support of each of the two hypotheses regarding the history of the virus will be examined.

First will be considered the hypothesis that JBE virus had been established on the island as a silent, endemic infection, for a few years at least, prior to the observed epidemic. The evidence in support of this is as follows:

1. As observed in the last column of Tables 6 and 9, dealing with native villages in the North and South, respectively, a number of persons, when bled in February or April, had neutralizing antibodies to JBE not associated with CF antibodies. Since CF antibodies in all *patients* were still present when bled between February and April, it could be assumed that *normal* persons without them were not infected during the epidemic. This interpretation of the serological findings is the simplest and is the accepted interpretation in work done in Japan, Okinawa and Korea, where the majority of previous JBE studies have been carried out.

2. The age distribution of clinical cases (Table 4), with a great predominance of cases under 20 years of age, is that observed in several areas of endemic virus activity. It is generally assumed that in an endemic area older children and adults are relatively spared because they have developed immunity earlier in life through inapparent infection.

3. Cases were not grouped in any time-geographical pattern so as to suggest that virus was newly introduced at one point and spread in a radial manner. Instead, the sequence was more compatible with an agent already present and widely distributed, which awaited suitable climatic or other conditions to manifest itself through increased infection rates.

Next for consideration is the opposite hypothesis, *viz.*, that JBE virus was newly introduced at some time shortly before the observed epidemic which began in November 1947 and that this was a true "virgin" epidemic. In support of this are the following findings:

1. No previous epidemic or series of suspected cases had ever occurred on this island in the native or a military population, both of which had had excellent medical care and surveillance by the American military forces for several decades. It is unlikely under such circumstances that all clinical activity would go unnoted. As discussed above, mumps virus in a dual etiological role was not essential to produce manifest, clinical encephalitis.

2. Although the earlier antibody tests made by Hodes on Guam were on a very small sample (four horses and four men), it is unlikely that, if conditions were the same then as in 1948, antibodies would be missed, particularly since we found all horses positive. Thus, Hodes' negative

TABLE 14*

*Morbidity rates per 1,000 for Japanese B encephalitis in Tokyo, Japan, 1948 (1,462 cases) and results of serological tests on normal Tokyo population by age groups, (September, 1948)*

| Age | Morb. rate | Serological tests | | | | | | Diff. % Pos.† Neut. & CF |
|---|---|---|---|---|---|---|---|---|
| | | Neutralization | | | Complement fixation | | | |
| | | No. tested | No. pos. | % Pos. | No. tested | No. pos. | % Pos. | |
| 0–4 | .85 | 48 | 10 | 20.8 | 55 | 8 | 14.5 | 6.3 |
| 5–9 | 1.17 | 59 | 20 | 33.9 | 68 | 8 | 11.8 | 22.1 |
| 10–19 | .43 | 153 | 87 | 56.9 | 160 | 9 | 5.6 | 51.2 |
| 20–39 | .20 | 88 | 78 | 88.6 | 93 | 8 | 8.6 | 80.0 |
| 40–59 | .09 | 71 | 65 | 91.5 | 62 | 1 | 1.6 | 89.9 |
| 60+ | .26 | 52 | 49 | 94.2 | 53 | 3 | 5.7 | 88.6 |
| Totals..... | .26 | 471 | 309 | 65.6 | 491 | 37 | 7.5 | 58.1 |

* Table adapted from Tigertt (1948).

† Per cent positive by neutralization less % positive by CF.

findings lend some support to the concept of the subsequent introduction of virus.

3. As pointed out above, the evidence is quite convincing that JBE virus disappeared from the island immediately following this epidemic. This strongly supports the hypothesis of its recent introduction, for if it had been adequately established to maintain itself for several years, it probably would have continued to maintain itself. Instead of this, some factor essential for endemicity appears to have been lacking. Furthermore, in no area of recognized endemicity of JBE virus where follow-up studies have been conducted, has the virus suddenly disappeared. Even SLE and WEE have failed to disappear in the Yakima Valley, Washington, in a temperate climate, after years of heavy vector population reduction through the use of tremendous amounts of highly effective agricultural insecticides including DDT (Reeves *et al.*, 1952). Furthermore, disappearance of virus from Guam cannot be attributed to mosquito-control measures, as can the disappearance of dengue. Evidence for this was previously reviewed.

4. The serological pattern in Guamanians from the southern villages differs distinctly from that seen in areas considered to be endemic. In Table 9, Section A, it will be noted that the percentage of individuals with neutralizing or CF antibodies does not vary significantly with age. The correlation between numbers of individuals possessing both types of antibodies is more evident in the February data in Table 9, Section B, than in the combined results, since by April there was considerable loss of CF antibodies.

These data should be compared with those of Table 14, based on a similar type of study made and reported previously (Tigertt, 1948) of a very large and explosive epidemic of JBE which occurred in Tokyo in 1948. In this outbreak 1,462 cases were reported, a rate of 0.26 per 1,000 (one-fourth that of Guam). At the end of the outbreak in September, serum samplings from several different areas of Tokyo were taken and tested for the two types of antibodies. The percentage with neutralizing antibodies increased in progressive age groups, while the number showing CF antibodies was maximal in the young and decreased sharply in the older age groups. It is believed that individuals with neutralizing antibodies (as a result of previous experience with the virus) who are again exposed to the virus seldom, if ever, develop CF antibodies. Thus, when sampled in the immediate postepidemic period, the differences by age group between percentages with positive NI and positive CF are a measure of previous experience with the virus.

The comparison between southern Guam and Tokyo is facilitated by Figure 5. It is clear that patterns of current infection and "previous" immunity were very different in Guam and in Tokyo; the latter an example of an endemic area. As a matter of particular interest, the infection rate observed in southern Guam of approximately 50 per cent is phenomenal and never before, to our knowledge, has such a rate been observed in an epidemic of any of the arthropod-borne viruses except for dengue. This again may be considered as some evidence to support the concept of a virgin outbreak. The infection rate

of 50 per cent just referred to is derived from the results of CF tests on 138 persons of all ages, bled in February, which showed 46.4 per cent positive (Table 9, Section B). Since this had fallen to 14.5 per cent by April there is a possibility that it was falling in February and may even have been above 50 per cent had a sample been taken in January. If all neutralizing antibodies resulted from current infection, which appears quite possible, the total infection rate was 80 per cent as indicated for the April bleeding.

5. Earlier in this report it has been noted that 11 of 27 clinical cases occurred in children under 5 years of age and only one case was seen in the over-20-year age group. Yet all age groups showed a very high rate of recent experience with the virus as manifested by CF antibodies. It is obvious that such a disease pattern may be readily explained in a population subject to equal exposure on the assumption that the young are more likely to show frank disease. In various laboratory animals also, differing age susceptibilities to viruses of this group are well known. In endemic JBE areas when faced with such an age distribution of clinical cases it has been asserted that the relative freedom from disease in the older age group was provided by the presence of neutralizing antibodies resulting from their prior experience with JBE virus. While this may well be true such an assumption should not be used to exclude the role of the age factor in the clinical response to initial infection. The prior experience may simply explain why such individuals do not develop much in the way of CF antibodies on re-exposure, and may be of little importance in explaining their relative insusceptibility to clinical disease.

The experience of American troops in Korea in 1950 is of interest. Hullinghorst *et al.*, (1950) found that 49 per cent of 149 nonvaccinated soldiers, evacuated for *other* than neurological causes, possessed JBE neutralizing antibodies after periods of exposure varying from 42 to 54 days. Further, the large majority of these men had NIs of 1,000 or more and a proportion had positive CFs. Thus, in Guam, the evidence of considerable experience of all age groups with the virus, with relative absence of clinical cases in adults, is entirely compatible with the concept of a virgin outbreak while, as noted in the preceding paragraph, the total serological pattern differs from that seen in known endemic areas.

6. The percentage of those with neutralizing antibodies minus those with CF antibodies in northern and southern villages, shown in the last columns of Tables 6 and 9, are remarkably similar, suggesting essentially identical experience with whatever produced the antibodies. Yet, the experience of these two areas in 1947–48 was entirely different near or at the end of the epidemic. It seems improbable on a small island like Guam that a virus endemically present and equally active in two areas (if these antibody findings are interpreted as "previous") would, under epidemic conditions presumably due to general climatic influence, suddenly become acutely epidemic in only *one portion* of the island. It seems more probable that the lack of uniformity during the epidemic is due to the recent entrance of the virus through some central or southern portal.

7. Dengue or similar viruses could hypothetically explain all the serological findings considered to have been the result of pre-epidemic infection. Or, as pointed out above, the serology could be explained in part, or even altogether, by an extremely rapid loss (2 or 3 months duration) of CF antibodies by many persons with inapparent JBE infection. That the fall in CF titer may be rapid is indicated in Table 9, Section B. A decrease occurred in CF antibodies from 46 per cent in February to 14 per cent in April. Considering that all persons with CF antibodies were infected during the current outbreak, this represents a reversion of about 66 per cent of the positives during a period of about 2 months. This is markedly different from what was observed in patients. On a limited scale, however, rapid loss of CF antibody associated

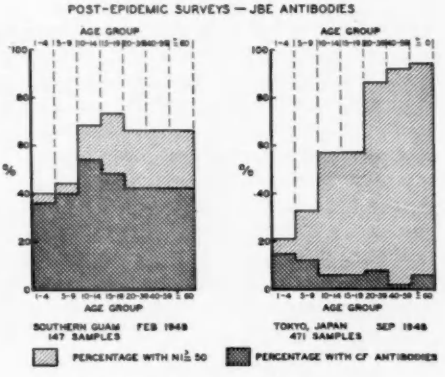


FIG. 5. *U. S. Army Photograph*

with inapparent infection has been observed previously by the authors.

Although this one explanation might be adequate, the dengue consideration is of interest. As reviewed above in the section on the interpretation of serology, there is no evidence that infection of man with one dengue virus will produce JBE neutralizing antibodies in a titer which could cause confusion. However, low neutralizing antibody titers have been observed in an occasional experimental monkey (Sabin, 1950). Nothing is known about what happens to the serological reactions with JBE virus in man after numerous infections with the various immunologic types (two known to date) of dengue virus. Furthermore, such repeated infections would not have occurred in children less than 5 years of age, for dengue viruses had disappeared at least 1 or 2 years before children of this age group were bled. Thus, the oldest of these were too young to have had this amount of experience. This group was found free of JBE neutralizing antibodies without associated CF antibody, in both areas. The dengue-antigen-overlap possibility has an additional attractive feature in interpreting the findings in this epidemic. Thus, because older persons possess the neutralizing antibodies, these older persons may have had some protection against clinical JBE, possibly explaining the age distribution of cases which is so near to that expected in JBE endemic areas. However, we must admit that we have no reliable information regarding what age distribution to expect in a "virgin" outbreak.

It appears quite unlikely that a virus other than dengue was responsible, unless it was transmitted only by *A. aegypti*, since sera from all children born subsequent to 1947, including many who were 9 years of age when bled, have been free from detectable JBE antibody. Furthermore, quantitative tests for neutralizing antibodies to WN, SLE and MVE, whenever made, always gave titers below those of JBE. This would indicate quite clearly that neither these viruses nor any other agents more closely related to these than to JBE, were responsible. Tests for several other group B viruses made on sera collected in 1953 were all negative except in the age group positive to dengue. Dengue antibodies were regularly present in association with these antibodies, suggesting that they were the result of dengue infections.

Thus, we conclude regarding the history of JBE virus on Guam, that the virus was certainly of relatively recent implantation and our choice between believing it was introduced a few years before or just before is inclined towards the latter. This leads to some speculation as to just how and when the virus was introduced.

That Guam was relatively isolated prior to the war is attested to by the infrequent introduction of mumps, measles, chicken pox and pertussis which all had had long periods of absence from the island. Further evidence of Guam's isolation is the fact that prior to World War II no *Anopheles* mosquito had ever been found in the Marianas Islands and that *A. albopictus*, present on adjacent Saipan, had never become established on Guam. In fact, only eight species of mosquitoes were known to be present on this lush tropical island while well over 50 are found on Luzon in the Philippines. However, during and after the war, traffic by air and water increased tremendously. In 1948 our entomological team found *Anopheles subpictus indefinitus* and *A. albopictus*, both having been introduced and readily established in recent years. Traffic was heavy with Japan and other much nearer areas where JBE virus is now known to be endemic. Mumps, measles, chicken pox and pertussis were all introduced about this time. Therefore, the introduction of JBE during this early post-war period appears quite probable, but whether by mosquito, man, domestic animal or bird would be difficult to judge. All seem quite possible.

*Possible vertebrate reservoirs.* The very small number of domestic mammals in 1947–48 (Table 11) and the high rate of immunity among them at the end of the epidemic (Table 12) would render them quite unsuitable as a reservoir for continued mosquito infection. Further, their small number might be postulated as a reason for the disappearance of the virus. However, it seems improbable that a lack of domestic mammals was critical in determining the suitability of Guam for the development of an endemic situation. On Okinawa, where an endemic situation did continue to exist subsequent to the 1945 epidemic, the animal population was much lower than that of Guam. A large bird population existed and there was some evidence that a few species became infected (Table 12). Possibly one or more species of these served as temporary sources of mosquito infection.

Man, himself, may have played the major role. The extremely high infection rates in man (50 or possibly 80 per cent), since they are not ordinarily observed in relation to this group of viruses (except for dengue or urban yellow fever where man is the epidemic source of mosquito infection), might add support to this concept. Since information on this point is so incomplete, even in areas subjected to study over many years, it is not unexpected that the situation in Guam is not revealing. However, it appears unlikely that the lack of vertebrate hosts of a suitable number or species to permit maintenance of the virus cycle was the deciding factor in breaking the established infection chain.

*Data on other Pacific islands.* From the studies carried out to date little can be concluded regarding viruses belonging to the B complex insofar as other islands are concerned. It is highly unlikely that JBE virus was present on Saipan, since the serological findings were so different from those of Guam. It seems equally apparent that other agents with a common antigen, but not identifiable from the available data, are active on a number of the Pacific islands from which students came to the Guam school. These islands include Samoa and Ponape where other serological sampling was attempted, with results pointing to the presence of other agents.

## SUMMARY AND CONCLUSIONS

From December 1947 through March 1948 a severe epidemic of encephalitis, affecting natives, Americans and other nonresidents, occurred on Guam. The epidemic was found to be due in part to JBE virus and in part to mumps virus. This was the first recognized outbreak of JBE on Guam or in any other truly tropical area. Mumps was occurring in an unusual epidemic form for it had been absent from the island for 6 or 8 years, and from one part for about 17 years. A total of 1,647 cases were reported in natives, a rate of 66.6 per 1,000. The total encephalitis rates were 1.86 per 1,000 among natives and 0.15 among the military, with somewhat similar low rates among various groups of nonresident civilian personnel. JBE virus was isolated from four of five fatal cases.

By using serological tests in addition to virus isolations it was determined that, of those cases which could be specifically diagnosed, approximately one-third had been infected by JBE virus only, one-third by mumps virus only, and one-third more or less simultaneously with both viruses. Only six cases could not be classified. The time of the peak of the mumps epidemic and of that of JBE coincided almost exactly. All fatal cases had JBE virus infection, one had mumps also. Serological patterns were observed to vary considerably between individuals but certain common patterns were noted.

The native cases with JBE virus infection had their highest incidence under 5 years of age. Males were more frequently affected than females. Cases occurred predominately in the southern portion of the island where there were few Americans and other nonresidents.

Climatologic data were intensively studied. Attention was focused on possible associations between climate and the possible dissemination of virus by a mosquito vector. Several climatologic aspects were unusual but a marked prolongation of the normal rainy season may have contributed to the epidemic. Findings from mosquito surveys, together with other data, pointed to *C. annulirostris* as the probable vector. The climatologic conclusions are compatible with its known breeding habits.

Serological surveys were made at the end of the epidemic on a number of population groups and on wild and domestic vertebrates. Samples of native children were bled again a year later (1948–49) and again in 1953 and in 1957. Interpretation of the serology is difficult and has been influenced by certain events and observations summarized in part as follows. Dengue was endemic until the vector *A. aegypti* was virtually eradicated in 1945 and 1946; then dengue disappeared as a clinical disease. No further proven cases of JBE encephalitis and no epidemic of a similar disease occurred subsequent to the epidemic studied. No dengue or JBE antibodies were found in any children born subsequent to the epidemic, yet no mosquito population or species decrease has been recognized since the eradication of *A. aegypti*, which was prior to the encephalitis epidemic.

Serological findings at the end of the epidemic included the following salient features. Varying proportions of wild and domestic mammals and birds, as well as man, had developed neutralizing antibodies. In man there were more persons with neutralizing antibodies than with CF antibodies except below 5 years of age. This excess of those

with neutralizing antibodies increased with age up to adulthood. This same difference between the proportion with CF and neutralizing antibodies was observed in areas of low and high current infection rates. Total CF antibody prevalence rates in southern villages were essentially 50 per cent just at the end of the epidemic. Two to 3 months later they had fallen to 14 per cent. Neutralizing-antibody titers were highest to JBE virus, lower to WN and lowest or absent to SLE virus. Fewer were tested for antibodies to MVE and these were negative or much lower in titer than to JBE. Sera collected in 1953 were tested with several other group B viruses. The only positives were in persons born before 1947 and these persons also had antibody to dengue viruses. CF antibodies to other group B viruses were frequently present. When included, SLE antigen gave elevated serum titers more frequently than the other antigens, but usually lower than those with JBE. It was concluded that JBE and dengue viruses were probably the only group B agents necessary to consider and that no one had been infected with dengue for about 2 years before the encephalitis outbreak.

JBE neutralizing antibodies without CF were considered to be due to one of three possibilities: (1) recent JBE infection, following which CF antibody had persisted only 1 to 3 months and had just been lost; (2) repeated previous dengue infections; or (3) infection with JBE virus which had occurred during previous years. Either of the first two interpretations supported the hypothesis that JBE virus had just invaded the island, and the last, that the virus had been established there for at least a few years. All evidence has been weighed in support of these two hypotheses and it is suggested that JBE virus probably invaded the island *de novo* as did mumps, and that this was a "virgin" outbreak. The virus then disappeared, apparently due to the absence of some factor necessary to permit its maintenance at an endemic level. The age distribution of cases may be that expected in a virgin outbreak but, since this is the first virgin epidemic on record, the age distribution is accepted with caution. Dengue possibly afforded some clinical protection and may have influenced the age pattern. Infection rates with JBE virus in certain villages probably attained a level of at least 50 per cent or possibly even 80 per cent, figures never previously approached in other reported outbreaks. It was observed that although *A. albopictus* was introduced into the island and has replaced *A. aegypti*, dengue nevertheless did not persist or recur.

ACKNOWLEDGMENTS

After the etiology of the outbreak had been determined independently in the two laboratories of the authors this became a large co-operative project involving the Navy, Army and the University of California and subsequently, with the transfer of two of the authors, the University of Pittsburgh. Many persons made important contributions, two of whom are acknowledged above with the authors or in the following text. Other participants are referred to by reference to their previous or concurrent publications regarding this same outbreak. In addition, we wish to acknowledge the important roles played in the field investigations by the following persons, listed alphabetically: R. Adm. C. L. Andrus, MC USN, Commanding Officer Naval Medical Center, Guam; C. Ph. M. Harry Dressler, USN, U. S. Naval Medical Center Laboratory No. 926; Lt. Cmdr. Thomas M. Floyd, MSC USN, U. S. Naval Medical Center, Guam; Capt. W. L. LaCasse, MSC USA, 207th Malaria Survey Detachment, Kyoto; Cmdr. F. R. Philbrook, MC USN, CINCPAC, Pearl Harbor; Col. G. N. Schuhmann, MC USA, Surgeon's Office, FEC, Tokyo; and Col. J. U. Weaver, MC USA, Surgeon, Marianas Bonins Command.

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## BOOKS RECEIVED

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*Neomycin, Its Nature and Practical Application*, by S. A. Waksman and others. 412 pages, ill. Baltimore, Williams & Wilkins Co., 1958, $5.00.

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*Handbuch der Virusforschung*, ed. by Prof. Dr. C. Hallauer, Bern, and Prof. Dr. K. F. Meyer, San Francisco. 688 pages, ill. Vienna, Springer-Verlag, 1958, $5.25.

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*Tuberculosis in White and Negro Children*, Vol. 1, by Janet B. Hardy and Vol. 2 by Miriam Braily. 122 and 103 pages, ill. Cambridge, Harvard Univ. Press, 1958, $7.50 and $4.50.

# BOOK REVIEWS

**Host-Parasite Relations in Living Cells,** A Symposium Sponsored by the James W. McLaughlin Fellowship Program, University of Texas, Medical Branch, Galveston, Texas, April 27, 1956, compiled and edited by Harriet M. Felton, M.D. 1st edition, 245 pages, illustrated. Springfield, Ill., Charles C Thomas. 1957. $6.50.

This book reports a one-day conference designed to stimulate an interdisciplinary consideration of the general question of host-parasite relationships at the cellular level. Eight formal presentations, covering subjects ranging from the use of the electron microscope in the study of the host cell and the parasite to a summary of concepts of resistance of the parasite, comprise the body of the report, together with the discussions thereon. The participants are qualified to speak with authority —for example, R. Dulbecco and J. T. Syverton deal with "cytopathogenicity" and "immunity" from the viewpoint of the virologist. E. W. Dempsey and C. E. Georgi contribute papers on the role of the electron microscope. Other formal contributions are by J. H. Hanks, R. A. Good, R. J. Dubos, and M. G. Sevag. In the discussions the reader is left with the impression that time limitations curtailed exploitation of the process of cross-fertilization. While "answers" are usually unobtainable via the conference technique, "ideas" or suggestions for alternative research approaches are the fruitful end product. In this instance it is likely that the participants derived more of value than will the reader of the transcript.

Few reproductions in color illustrating viral cytopathogenicity are available; the value of those in the present book would be enhanced if the legends were clearly associated with the respective figures.

THOMAS H. WELLER

**World-Atlas of Epidemic Diseases,** Edited by Ernst Rodenwaldt and Helmut J. Jusatz, under the Sponsorship of the Heidelberger Akademie der Wissenschaften. First of four issues in the third part of the series. 36 pp., 10 maps. Hamburg: Falk-Verlag.

The first instalment of the third series of the atlas of epidemic diseases should be specially interesting to students of tropical medicine in this country because five of its ten maps illustrate disease conditions in the Americas, namely (a) plague in South America 1899–1953; (b) yellow fever in South America 1945–1954; (c) leishmaniasis in South America 1911–1955; (d) schistosomiasis mansoni in South America 1908–1953 and (e) filariasis in South America 1939–1955, while a sixth map shows the distribution of the population in South and Central America 1950/54. The remaining maps are devoted to brucellosis in Europe, 1929–1955; rabies in Africa, 1947–1953, and to the distribution of schistosomiasis hematobia and Schistosomiasis mansoni and their vectors around 1955 in Africa and the adjacent regions. As in the former issues of the Atlas these main maps, which are beautifully executed in colors, are accompanied by supplementary materials, consisting of black-and-white maps and tabulations as well as by explanatory articles in German and English.

In the article on plague in the New World accompanying the plague map, Raettig is not in favor of the view held by some American authors that the infection might have been introduced into the continent at an early date from Asia via the Behring Strait and Alaska, but accepts the commonly held belief in the importation of the disease by the sea-route from Hong Kong about 1900. Whether this was so is difficult to decide, the more so as plague had become entrenched at some points of the China coast well before it reached Hong Kong towards the end of the last century. Pointing to the successive involvement of the wild rodent populations in wide areas of America, Raettig stresses the necessity of "watchful and unceasing research and inspection" in the enzootic regions in order to avoid large outbreaks in the future. Desirable as such constant surveys undoubtedly are, one should not lose sight of the view held by most competent observers in this country that the adoption of strict anti-rat measures in the endangered human settlements goes a long way to protect them against at least major inroads of the infection.

The article on the recent incidence of yellow fever in America by Höring and Höring also strikes a gloomy note, stating that though, judging by the absolute figures of infection and deaths, the disease now appears to be of little importance in America as well as in Africa,

> "two circumstances must not be forgotten: firstly, that its decrease, particularly in South America, is due to preventive measures carried out with an enormous amount of expenditure and organization . . . and, secondly, that it appears to be increasing rather than decreasing in importance as a potential danger in the warm countries, running parallel to their cultural and economic development and especially with their expanding transport systems."

As the authors add, the occurrence of the disease

in Central America and Trinidad since 1948 served as a drastic example "of the staggering economic blows that can be dealt by such an outbreak in spite of the low loss of life today." One must hope, however, that the progress in wealth due to economic development and increased transportation facilities will also lead to progress in health, in this special case to intensified and improved methods of *A. aegypti* control.

R. POLLITZER

**International Sanitary Regulations,** World Health Organization, Amended 1956. 129 pp. Ill. Geneva, Switzerland, World Health Organization. 1957. Price, 5/; $1.00; Sw. fr. 3.

The Constitution of the World Health Organization, drafted in 1946, specifically provided that the World Health Assembly should ". . . have the authority to adopt regulations concerning . . . sanitary and quarantine requirements and other procedures designed to prevent the international spread of disease. . . ." At that time, even though there was a long tradition of agreements and conventions on international traffic, dating as far back as 1851, the situation was confused and unsatisfactory. A number of conventions were simultaneously in force and some of their provisions were obsolete and not based on current medical knowledge and caused serious disturbance to traffic and trade. An Expert Committee on International Epidemiology and Quarantine was established in 1948 and was instructed ". . . to revise the existing International Sanitary Conventions . . . into a single body of regulations covering the needs of all travellers. . . ." The Committee concerned itself only with six of the most serious communicable diseases (plague, cholera, yellow fever, smallpox, louse-borne typhus, and relapsing fever) and prepared the International Sanitary Regulations which were adopted in 1951. The Regulations were amended in 1955 in respect to the yellow fever clauses—a subject of considerable controversy—and in 1956, when the transitory measures for control of pilgrim traffic were deleted and the existing international certificate of vaccination against smallpox was modified.

The present volume contains the Regulations as amended, and it is divided into ten parts. Part I gives the definitions of the terms used. Part II refers to the procedures for notification. Part III establishes the sanitary organization required in ports and airports for the application of the measures provided. Part IV deals with the sanitary measures to be applied on departure or arrival as well as the measures concerning the international transport of goods, baggage and mail. Part V contains the specific provisions relating to each of the six quarantinable diseases. The remaining five parts deal with subjects such as a list of 12 previous Sanitary Conventions which are replaced by the Regulations, the mechanism to settle disputes between states, etc.

In a series of appendices, blank forms of the international documents required (vaccination certificates, declaration of health by shipmasters) are presented. A slight modification of the international certificate of vaccination against smallpox previously used allows for the inclusion of the date of the reading as a successful or unsuccessful vaccination. This is a definite improvement which greatly facilitates the handling of contacts.

In an annex, the position of 180 states and territories as regards the Regulations is given. Of these, 142 have accepted without reservations the 1951 Regulations and 117 are bound by them as subsequently amended. This is really a remarkable achievement considering the complexity of the problem and the fact that there are still some controversial subjects. In other annexes, useful information such as a list of the yellow fever receptive areas and procedures for the disinsectization of aircraft is given.

A definite improvement of this volume over its predecessors is that the various articles are annotated to show states and territories that have submitted reservations, the text of which is reproduced in an annex. Another useful feature is the inclusion in footnotes, under the articles to which they refer, of the various observations and interpretations of the Expert Committee on International Quarantine in its annual review of the functioning of the Regulations.

In conclusion, this publication should be most useful in meeting the requirements of port and airport health oficers for which it is primarily designed.

MARIO PIZZI

**The Incurable Wound,** by Berton Roueché. 177 pages. Boston; Little, Brown and Company, 1958. $3.50.

The Incurable Wound is a collection of narratives of medical detection, designed for laymen and first appearing in the "New Yorker" since 1954. "The incurable wound" relates to rabies contracted through bats, and is the most dramatic of the six tales. Others relate to amnesia, aspirin, poison control centers and ACTH. In all, for the unintiated these are, or should be, truly fascinating. But for those who have as their daily tasks the very substance of these tales they provide little that is new.

HAMILTON H. ANDERSON

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